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EDITED BY
Gillian Eggleston and Gregory L. Cote

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Oligosaccharides in Food and Agriculture

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS Books Department

Preface

Oligosaccharides in food and agriculture are currently garnering much attention, especially in their application as prebiotics (non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon). This attention, coupled with new and exciting developments in the separation and analysis of oligosaccharides, the development of numerous commercial enzymatic processes to produce oligosaccharides from polysaccharides, as well as the discovery of new roles for polysaccharides in plants (e.g., as markers for plant deterioration), are the primary reasons that spurred the two editors' interests in producing this book and the associated American Chemical Society (ACS) Division of Carbohydrate Chemistry symposium *Oligosaccharides in Food and Agriculture.* This symposium was held April 2002 in Orlando, Florida at the 223rd ACS National Meeting

The objective of this book is to provide an overall view of the current understanding of the occurrence and function of oligosaccharides in food and agriculture. The book focuses mainly on oligosaccharides derived from natural plant sources, in particular those found in or produced from agricultural commodities.

The chapters are arranged so as to provide the reader with an understanding of recent developments in the separation and analyses of oligosaccharides. This is then followed by prebiotic oligosaccharides and includes many types and sources for them. The final sections of the book contain chapters on novel primary and secondary oligosaccharides, as well as those naturally occurring or derived from the microbial or enzymatic reaction on plant matter.

In addition to chapters written by the symposium speakers, other chapters were contributed by researchers who were invited to provide a more encompassing view of this exciting and rapidly developing field. Their chapters include information ranging from isomaltooligosaccharides to prebiotic oligosaccharides from waste biomass.

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It was a truly cooperative effort by the two editors to pull these chapters together into a comprehensive text. With worldwide contributing authors, the editors feel that the text should provide the readers with an up-to-date review of this rapidly changing field.

We acknowledge the team of distinguished reviewers who made this book possible by their thorough and professional reviews: James BeMiller, William Burgess, Michael K. Dowd, Mary An Godshall, Scott M. Holt, Gerald Nagahashi, Peter Reilly, John F. Robyt, Randal L. Shogren, George A. Somkuti, Bernard Y. Tao, and John R. Vercellotti.

We are also indebted and deeply appreciate the symposium sponsors without whose donations to the symposium and book could not have been realized: ACS Division of Carbohydrate Chemistry, Archer Daniels Midland Company, Imperial Sensus, Kabo Chemicals and Agriculture, Inc., Megazyme, and Neose Technologies, Inc. We thank the authors for their contributions to this book and we hope the readers will enjoy the book.

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Chapter 1

Oligosaccharides in Food and Agriculture

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An overview of oligosaccharides in food and agriculture is presented. Oligosaccharides are carbohydrates generally consisting of two to ten monomeric residues linked by *O*-glycosidic bonds. The wide occurrence and function of oligosaccharides in plants is reported. Up-to-date techniques for the separation and analysis of oligosaccharides are reviewed. Commercial applications in the fields of nutrition and food ingredients are discussed, with emphasis on prebiotic and cyclic oligosaccharides. Sources of oligosaccharides include those from insect or microbial action on plant material. Oligosaccharides as plant regulatory molecules are described. Finally, the future outlook for oligosaccharides in food and agriculture is discussed.

Introduction

Oligosaccharides may be produced synthetically or via microbial fermentative and enzymatic processes, or they may be derived from naturally occurring sources such as plant matter. Although the role of oligosaccharides in animal cell surfaces

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is extremely important and has attracted much attention from synthetic chemists for medical applications, we will focus on oligosaccharides derived from natural plant sources, in particular those which may be found in or produced from agricultural materials. Oligosaccharides currently produced for commercial markets include cyclomaltodextrins, maltodextrins, fructooligosaccharides, galactooligosaccharides, soy oligosaccharides, and others produced mainly for the prebiotic markets in Japan and Europe.

Enzymatic processes are involved in the production of virtually all agriculturally important oligosaccharides, whether for food or industrial use; therefore, any discussion of oligosaccharides will invariably include studies on enzymes. In the case of maltodextrins and cyclomaltodextrins, enzymes are responsible for the conversion of polysaccharides, such as starch, into oligosaccharides. In other cases, such as certain fructooligosaccharides, microbial or plant transglycosylases may be involved. Most recently, reversion reactions of glycosidases and their genetically engineered derivatives, glycosynthases, may be used for oligosaccharide synthesis.

This chapter is intended to give some examples of current research in the separation and analysis of oligosaccharides and in the synthesis and applications of oligosaccharides that are currently, or are expected to become, important in the food industry and agriculture.

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Nomenclature and Classification of Oligosaccharides

It is generally agreed, within the international carbohydrate community, that an oligosaccharide is a carbohydrate consisting of 2-10 monosaccharide residues linked by *O*-glycosidic bonds (*1*), although some consider carbohydrates with 2-20 monosaccharide residues as oligosaccharides (*2*). For a full description of the nomenclature of oligosaccharides the authors refer you to the most recent IUPAC and IUMB recommendations (*3*).

Many of the known plant oligosaccharides have established names which were often assigned before the structures were known (*4*) and, therefore, give little information about the structure. In order to clarify the often confusing multiplicity of plant oligosaccharides, Kandler and Hopf (*4*) grouped them into two distinct classes: primary and secondary oligosaccharides, and this classification will be used in this chapter. Primary oligosaccharides are those synthesized *in vivo* from a mono- or oligosaccharide and a glycosyl donor by the action of a glycosyl transferase (*4*). Sucrose is the most common primary oligosaccharide in plants. Secondary oligosaccharides are those formed *in vivo* or *in vitro* by hydrolysis of higher oligosaccharides, polysaccharides, glycoproteins, and glycolipids.

Extraction, Separation, and Analysis of Oligosaccharides

Oligosaccharide extraction from freshly harvested plant material must be undertaken immediately, or the material must be stored either below -20°C, or freeze-dried, in order to prevent enzymatic or chemical degradation. Extraction of oligosaccharides is often performed using 70% alcohol, but for many modern analytical techniques, plant materials can be analyzed directly and, therefore, there is no need for cumbersome extraction procedures.

Despite the trend to more sophisticated (and expensive) instrumental methods for the analysis of oligosaccharides, the traditional methods of paper chromatography and thin-layer chromatography (TLC) still have their place. Thin-layer chromatography, in particular, is still a useful technique for the rapid separation of large numbers of samples, requiring little or no prior clean-up (*5*). For screening studies, TLC may be used to analyze hundreds of samples in a single day. It has the added advantage of relatively low cost, as no expensive instrumentation is necessary. Developed plates may be scanned using ordinary desktop scanners and analyzed using densitometry software, yielding quantitative results that are perfectly acceptable for many purposes (*6-8*).

High performance size exclusion chromatography (HPSEC) and gel permeation chromatography (GPC) with laser light scattering (LLS) or refractive index detection allow the separation and direct detection of oligosaccharides and provides molecular weight distribution information. However, laser light scattering can sometimes give erroneous results, because of molecule-molecule interactions and associations which can occur in higher MW oligosaccharides, and refractive index detection of oligosaccharides is very insensitive (detects only in the mg and hundreds of μg range)

Oligosaccharides can be separated by high performance liquid chromatography (HPLC), using reversed phase columns, amino-silica columns, or ion-exchange sulfonate resin columns with calcium, lead, or silver counterions. High performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) is now frequently used to separate and directly detect oligosaccharides at alkaline pH using gradient methods. HPAEC offers high separation resolution of oligosaccharides and even oligosaccharide isomers, coupled with very sensitive detection (2). However, the mass sensitivity of PAD decreases with an increase in degree of polymerization (DP) (9).

High performance capillary electrophoresis (HPCE) with laser-induced fluorescence (LIF) detection also provides high resolution of oligosaccharides, but a pre-column derivatization is required to produce spectroscopically active compounds. HPCE has also been coupled with PAD to analyze oligosaccharide and alditol mixtures (*10*).

McPherson and Jane (*11*), using HPAEC-PAD on enzyme-digested starches were able to detect maltooligosaccharides up to DP 85. In comparison, in a recent

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comparative study of oligosaccharides by Kuhn *et al.* (*12*) using capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) and HPAEC-PAD, dextran oligosaccharides up to 45 DP were detected by HPCE and HPAEC-PAD, whereas MALDI-TOF MS allowed detection from DP 4 to DP 60. HPAEC-PAD was observed to be the most sensitive technique, but the separation resolution performance was better in HPCE and MALDI-TOF MS. Another advantage of MALDI-TOF MS is that it can provide accurate mass values, and the exact number of monosaccharides present in an oligosaccharide can be found. Conversely, a disadvantage of MALDI-TOF MS is that it is a destructive technique and, therefore, preparative work cannot be undertaken.

Fluorophore-assisted carbohydrate electrophoresis (FACE) technology is also used to separate and detect oligosaccharides, particularly from glycoconjugates (*13*). Analysis involves four steps: release, labeling with a fluorescent tag, separation using precast polyacrylamide gels, and imaging. Although FACE technology is simple and reliable, its use is not yet widespread. This may be due to a lack of papers describing specific applications, or possibly due to unfamiliarity with the technique.

Another technique also used to separate and detect oligosaccharides is the automated use of modern planar chromatography (*14*). This utilizes high performance thin-layer chromatography plates, automated multiple development, completely automated elution systems, and sample positioning and spots detection apparatus, all of which allows reproducible separation of different oligosaccharides and other compounds in complex mixtures. Despite the improvement of TLC and HPLC techniques, gas chromatography (GC) still continues to have a place in oligosaccharide analysis, particularly for structural studies, although pre-derivatization is required.

Nuclear magnetic resonance (NMR) is a powerful technique used to elucidate the structure of oligosaccharides (*15-16*), especially with the advent of 2-D (*17*) and various hyphenated methods for the determination of position and configuration of glycosidic linkages (*18*).

Many of the separation and analytical techniques mentioned above are used in conjunction with enzymatic or chemical degradation of an unknown oligosaccharide. A typical structural analysis of a complex oligosaccharide may involve partial acid hydrolysis or hydrolysis by specific enzymes, followed by separation and identification of the hydrolytic products. This combination of methods often yields structural information that cannot be obtained by methylation analysis, NMR, or chromatographic techniques alone. In particular, monomer sequence and distribution of branches and other substituents may be obtained in this way.

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Oligosaccharides as Nutrients and Food Ingredients

Prebiotics

Prebiotics have been defined as nondigestible food ingredients that beneficially affect the animal host by selectively stimulating the growth of certain bacteria in the colon which are advantageous to the host (*19*). They typically do this by serving as selective substrates for so-called probiotic bacteria. Most prebiotics are oligosaccharides, the best-known example being the β-(2→1)-linked fructooligosaccharides (FOS). These fructooligosaccharides can be derived from inulin (*20*), or synthesized from sucrose via fungal fructosyltransferases (*21*). Fructooligosaccharides with other linkages, such as β-(2→6), can be produced from sucrose by thermolysis (*22*), or through the action of levansucrase (*23*).

Many other nondigestible oligosaccharides are also used as prebiotics, or are being investigated as potential prebiotics. These include oligosaccharides of D-galactose, D-glucose, D-xylose, *N*-acetyl-D-glucosamine, and combinations thereof (*24-25*).

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Naturally Occurring Plant Oligosaccharides

Primary Oligosaccharides

Apart from sucrose itself, primary oligosaccharides in plants are mainly composed of glucose oligosaccharides, oligosaccharides based on sucrose, and oligosaccharides containing polyhydric alcohols (*4*). The vast majority of primary plant oligosaccharides are those based on sucrose and most attention will be given to them here. Examples of glucose oligosaccharides include α,α-trehalose (*O*–α-D-glucopyranosyl-(1↔1)-α-D-glucopyranoside) and selaginose (*O*-α-D-glucopyranosyl (1→2)-*O*–α-D-glucopyranosyl-(1↔1)-*O*–α-D-glucopyranoside), which serve as main soluble reserve carbohydrates in pteridophyte plants. An example of an oligosaccharide containing a polyhydric alcohol is the well known galactinol (*O*-α-D-galactopyranosyl-(1→1)-*myo*-inositol) which serves as a D-galactosyl donor in the biosynthesis of raffinose and its higher homologs.

Primary oligosaccharides based on sucrose are formed by the transfer of D-galactopyranosyl, D-glucopyranosyl, or D-fructofuranosyl residues to sucrose (*4*). Series or families of homologous primary oligosaccharides exist, of which the "raffinose" and "fructan" series are the best known and most widespread. Another series is the "lychnose" series (lychnose: *O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranosyl-(1←1)-α-D-galactopyranoside) which is described by Kandler and Hopf (*4*).

The raffinose series of oligosaccharides is shown in Fig. 1. The trisaccharide raffinose is formed by the addition of D-galactose to the D-glucose moiety of sucrose via an α–(1→6) linkage. The series is formed by the continual attachment of a galactose residue via an α–(1→6) linkage to the galactose moiety of the previous oligosaccharide in the series (Fig. 1) and continues up to a nonasaccharide. These oligosaccharides are important carbohydrate reserves in the vegetative storage organs and seeds of many plants (*26*), and also play an important role in sugar translocation.

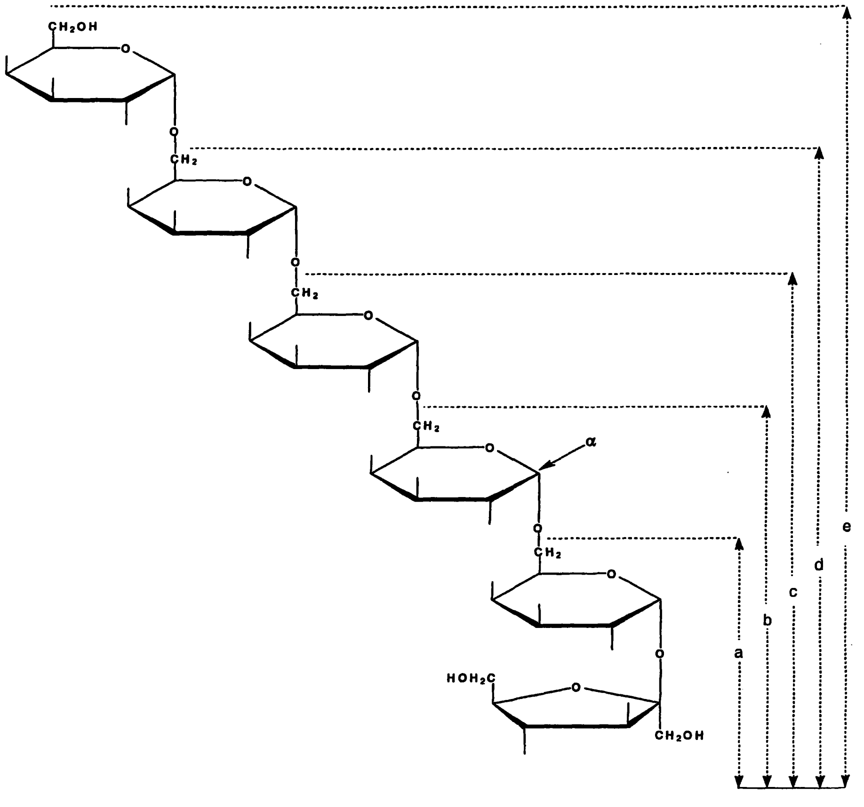

Figure 1. Raffinose series of oligosaccharides, (a) sucrose, (b) raffinose, (c) stachyose, (d) verbascose, (e) aungose.

The fructan or kestose series of oligosaccharides have been found in at least 12% of vascular plant species (*27*). Fructans contain one glucose moiety per oligosaccharide or polysaccharide chain, derived from the parent sucrose molecule. The simplest fructan, monofructosyl sucrose is a trisaccharide and exists as three isomers: 1-kestose (1^F-β-D-fructofuranosylsucrose), 6-kestose^F (6 -β-D-f-

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ructofuranosylsucrose) and neo-kestose (6^G-β-D-fructofuranosylsucrose). These isomers form the basis of three major fructan sub-series with different linkage patterns. The 1-kestose (or iso-kestose) series is well characterized and is found particularly in tubers of *Helianthus tuberosus*; these fructans consist of linear nonreducing chains with β-(2→1) linkages and are known as inulins. They have become very popular commercially for food and medicinal purposes and recent studies and applications are in further chapters of this book. The 6-kestose series of fructans consist of adjacent residues linked by β-(2→6) bonds; they are often referred to as levans or as phleins (*28*). The third series of fructans is the neo-kestose series. Neo-kestose differs from the other two trisaccharide isomers in that the D-glucose moiety is linked directly to two fructose moieties through the 1 and 6 carbon positions. As a consequence, chain elongation can occur on both D-fructose residues which results in a linear, nonreducing polysaccharide containing a D-glucose residue within the polysaccharide chain (*28*). Fructans containing different isomers and linkages within the same chain and branched fructans have also been described (*28*), and mixtures of fructans with different structures within a single plant species may be quite common (*28*).

Secondary Oligosaccharides

The most reported secondary oligosaccharides that are formed *in vivo* from the hydrolysis of primary oligosaccharides are melibiose (*O*-α-D-galactopyranosyl-(1→6)-D-glucose) and gentiobiose (*O*-β-D-glucopyranosyl-(1→6)-D-glucose). Melibiose is a breakdown product of raffinose in which the D-fructose unit has been removed. Gentiobiose is likewise a breakdown product from gentianose (*O*-β-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl(1↔2)-β-D-fructofuranoside).

When reserve polysaccharides are mobilized by plants, they are broken down by various endo- and exohydrolases, resulting in a series of secondary oligosaccharides and ultimately monosaccharides. Only small quantities of these secondary oligosaccharides are found in plant tissue during mobilization (*4*). The most common of these are maltose (*O*-α-D-glucopyranosyl-(1→4)-D-glucose) from the action of amylase on starch, mannobiose (*O*-β-D-mannopyranosyl-(1→4)-D-mannose) from either mannan in the seeds of dates or from galactomannans in leguminous seeds, and inulobiose (*O*-β-D-fructofuranosyl-(1→2)-D-fructofuranoside) derived from fructan (*4*).

Secondary oligosaccharides can also be formed *in vitro* by hydrolysis of higher oligosaccharides and polysaccharides, usually with dilute mineral acids or hydrolases. Such oligosaccharides include cellobiose (*O*-β-D-glucopyranosyl-(1→4)-D-glucose) the basic repeating unit of cellulose, and isomaltose (*O*-α-D-glucopyranosyl-(1→6)-D-glucose), the branching unit of amylopectin. Series of

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higher homologues may also be formed in this way. See Karrer *et al.* (*29*) for a review.

A very important class of secondary oligosaccharides derived from plant polysaccharides consists of those arising from the hydrolysis of starch (*30*). Starch is widespread in plants, and is used not only by the plants themselves as a food reserve, but is also utilized by most animals and a myriad of fungi and microbes. Most starches are mixtures of two polysaccharides, amylose and amylopectin. Amylose is composed of linear chains of 4-*O*-α-D-glucopyranosyl units, whereas amylopectin contains branching at *O*-6 of the 4-*O*-α-D-glucopyranosyl backbone chains. Various enzymes known generically as amylases break starch down into oligosaccharides and D-glucose. If these oligosaccharides contain exclusively 4-*O*-α-D-glucopyranosyl units, they are referred to as maltodextrins, based on their structural similarity to maltose. Branched oligosaccharides also arise from the branch points in amylopectin. As a general rule, maltodextrins tend to be readily digestible and form a significant proportion of the caloric intake of most herbivorous and omnivorous animals. However, the branched oligosaccharides may or may not be digested, depending on the activity of the enzymes present. Those that pass through the upper digestive tract can reach the colon and serve as substrates for fermentative microbes. In fact, certain mixtures of branched oligosaccharides derived from starch hydrolyzates, such as panose and isopanose, are being studied for their prebiotic activity.

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Oligosaccharides from Breakdown of Polysaccharides

The secondary oligosaccharides mentioned above exist primarily within the plant and result from breakdown *in vivo* by endogenous enzymes. Polysaccharides may also be broken down *in vivo* by exogenous digestive enzymes, particularly those of microbial origin, and can be broken down *in vitro* by mineral acids. The conversion of biomass and much of the carbon cycle depends on the enzyme-catalyzed degradation of polysaccharides to monosaccharides, often through oligosaccharide intermediates. As discussed in the preceding section, cellulolytic and amylolytic enzymes hydrolyze cellulose and starch, respectively, yielding series of glucooligosaccharides as the first step in their conversion to D-glucose. Nearly any polysaccharide may be broken down in similar manner; the two most common enzymes involved are endohydrolases and exohydrolases. It is typical for endohydrolases to first break down a long polysaccharide chain into short oligosaccharides, followed by hydrolysis of the oligosaccharides to monosaccharides by exohydrolases. Other enzymes may also be involved, including debranching enzymes, acetyl or feruloyl esterases, phosphorylases, transferases, etc.

Applications of several of these enzymatic processes have been developed. The best known and most widespread is undoubtedly the hydrolysis of starch. Starch may be converted to glucose by a combination of amylolytic hydrolases, followed by isomerization to yield high-fructose corn syrup, a product which is now widely used in the beverage industry. Mixtures of maltodextrins may also be obtained by enzymatic hydrolysis of starch. Vandamme and Soetaert (*31*) have reviewed other enzymatic modifications of polysaccharides, including hydrolysis to oligosaccharides. One example is the use of endo-mannanase to convert guar gum to a mixture of low-molecular weight galactomannan oligomers, used as a soluble dietary fiber and prebiotic. Besides simple hydrolysis, a combination of hydrolysis and transglycosylation may also yield unusual oligosaccharides. Trehalose is manufactured in this way, using a unique enzymatic system that converts starch to this useful nonreducing disaccharide (*32*). Trehalose is used as a cryoprotectant in the presevation of biological material, and has also been marketed for use in cosmetics, pharmaceuticals, and foods (*32*). Cyclic oligosaccharides may also be produced enzymatically from starch.

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Cyclic Oligosaccharides

An unusual and interesting class of oligosaccharides is those in which the glycosidic chain closes back upon itself, so that there is no reducing end (*33*). These molecules can contain as few as two monosaccharide units, in the case of difructose dianhydride (*34*), or much longer chains of twenty or more monosaccharide units (*35-37*). The best known of these are the cyclomaltodextrins, also known as cyclodextrins, cycloamyloses or Schardinger dextrins after their discoverer (*38*). These cyclic maltodextrins typically contain six, seven, or eight D-glucose units (known as α, β, and γ-cyclomaltodextrin, respectively), although larger ones have been isolated and described. Their most useful characteristic is the presence of a hollow cavity in the middle of the macrocyclic ring. The interior of this cavity is surrounded by a relatively hydrophobic surface, so hydrophobic molecules are able to enter the space and form stable inclusion complexes. This is useful for the formation of water-soluble complexes of hydrophobic molecules, particularly drugs, as well as oil-based flavors and fragrances. Cyclomaltodextrins are also useful in deodorizing sprays and treatments, since they are able to complex and thus neutralize odoriferous compounds. Cyclomaltodextrins with side chain branches are more water soluble, which enhances the solubilization process (*39*). Cyclomaltodextrins are produced from starch or maltodextrins by the action of bacterial enzymes known cyclomaltodextrin glucanotransferases (CGTases) (*40*). Numerous applications for cyclomaltodextrins exist, based mainly on their ability to form inclusion complexes (*41-42*), and interest in commercial production has led to the cloning and expression of CGTase in potatoes (*43*).

Many other naturally occurring cyclic oligosaccharides are also known, ranging from difructose dianhydrides and cyclic glucotetrasaccharide (*44*) to large β-(1→2)-linked D-glucans produced by various Rhizobial strains (*45*).

The Occurrence of Oligosaccharides from Insect or Microbial Action on Plant Material

Certain oligosaccharides occur in or on living plant tissue attacked by insects or infected with microbes (bacteria, yeasts, and fungi), as well as from postharvest plant material that has deteriorated from microbial reactions.

The greatest source of oligosaccharides from plant material attacked by insects is honey. There are two types of honey: floral and honeydew. Floral honey originates from the nectar of flowers. Honeydew is obtained by the honeybee indirectly from sweet syrups excreted by various hemipterous insects feeding on tree phloem sap. Most of the honey oligosaccharides are produced by enzymes present in the intestines of bees from nectar containing sucrose, glucose, and fructose or honeydew (*4*). The essential difference between the composition of nectar and honeydew is that honeydew contains significant quantities of the oligosaccharides melezitose (*O*-α-D-glucopyranosyl-(1→3)-β-fructofuranosyl-(2↔1)-α-D-glucopyranoside) and erlose (*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside). The review of Doner and Hicks (*46*) is recommended for a list of the unequivocally identified oligosaccharides in honey.

Microbial reactions are responsible for the deterioration of much plant material, particularly post-harvest plant material, and some of these deterioration reactions can lead to the formation of oligosaccharides. Two of the best examples of oligosaccharide formation on plant microbial deterioration in agriculture are associated with the industrial cultivation and processing of sugarbeet and sugarcane.

Both sugarbeet and sugarcane varieties, especially those damaged by frosts, diseases or pests, are highly susceptible to deterioration by dextran forming bacteria, particularly *Leuconostoc* spp. Dextransucrase, secreted by the bacteria, catalyzes the formation of dextran and associated oligosaccharides (*47-48*) from sucrose. Recently, some of these oligosaccharides, have been put forward as indicators of both beet (leucrose *49*) and cane (isomaltotriose *50*) preharvest and postharvest deterioration.

Kestose trisaccharides are also present in both sugarcane (*51*) and sugarbeet (*52*) plants. Morel du Boil (*53*) showed that kestose trisaccharides are responsible for sucrose crystal elongation and deformation in the factory crystallization process. Kestose trisaccharides can increase dramatically in the postharvest deterioration of cane, particularly whole-stalk cane under dry conditions (*54*), which is most likely

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caused by enzymatic reactions by invertases from the cane and/or yeasts. Kestoses were also reported (*55*) to increase on the storage of beets.

Other Roles and Sources

In the 1980s and 1990s (*56-57*) researchers at the Complex Carbohydrate Research Center in Athens, Georgia, USA, reported a newly observed role of oligosaccharides: their ability to act as plant regulatory molecules. Oligosaccharides with regulatory activities are referred to as oligosaccharins (*56*). Plant regulating activities include defense against pathogens and host plant-pathogen interactions, as well as the ability to regulate plant growth and development processes (*56*). Several plant cell-wall derived oligosaccharides have been identified that have growth-regulating activity (*58*) like plant hormones such as auxin and gibberellin. For example, fucosylated xyloglucan derived oligosaccharides have been shown to inhibit endogenous plant growth and growth stimulated by gibberellic acid (*59*). Oligosaccharides derived from galactomannan inhibit auxin-induced pea stem growth *in vitro* (*60*). Pectic and hemicellulosic oligosaccharides are known to regulate the development processes of fruit ripening and organ morphogenesis (*56*).

Another source of oligosaccharides is those formed from thermal degradation reactions of agricultural commodities. For example, Manley-Harris and Richards (*22,61*) demonstrated that kestose trisaccharides could be produced from the pyrolysis of crystalline and amorphous sucrose.

Future Outlook

Oligosaccharides in food and agriculture are currently garnering much attention, especially in their application as prebiotics. Much remains to be learned about the mechanism of action of prebiotics, in particular the complex interactions among carbohydrate substrates, mixed populations of microbes, and various host environments under a variety of dietary influences. However, the authors expect this attention to continue as new oligosaccharides are developed for use in so-called functional foods. It is likely that these oligosaccharides will be produced using microbial or enzymatic systems, which will necessitate further research on transglycosylation reactions by a wide variety of enzymes. Glycosyltransferases, glycosidases, and genetically engineered “glycosynthases” will play a role in this rapidly developing field.

Cyclomaltodextrins, although known for over a century, have only in the past twenty or thirty years found commercial applications. It is expected that this trend will continue, as more derivatives of cyclomaltodextrins find applications in consumer products, medicine, and industry. Other cyclic oligosaccharides will

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probably find other specialized uses as well. Cyclic dextrans (*62*) and their derivatives (*63*) possess unique properties that are currently under investigation in Japan for medical applications. A cyclic tetrasaccharide that can be synthesized enzymatically from starch or sucrose (*44,64*) is also being produced on a pilot scale in Japan. The physiological role these and other cyclic oligosaccharides play in nature will eventually become more clearly understood (*45,64*).

Over the last two decades new roles for oligosaccharides in plants have been discovered, particularly their role as plant regulatory molecules. More and different roles are expected to be discovered in future years.

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Chapter 2

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection for the Determination of Oligosaccharides in Foods and Agricultural Products

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HPAE-PAD is a well-established technique for determining both neutral and anionic oligosaccharides and polysaccharides from plant and animal sources. The high resolution of HPAE and the high sensitivity of PAD allow oligosaccharide determinations without pre- or post-column sample derivatization. With these properties, HPAE-PAD can determine higher degrees of polymerization of a carbohydrate polymer (e.g. amylose) than other techniques, and is able to determine a small amount of one oligosaccharide in the presence of a large amount of other carbohydrates. These two applications and the ability to profile complex mixtures of oligo- and polysaccharides have established HPAE-PAD as an important technique for analyzing carbohydrates in food and agricultural products. This chapter will review the basic separation and detection principles of HPAE-PAD, with an emphasis on the separation properties and peak area responses of oligo- and polysaccharides. These principles are applied to both describing and designing determinations of oligo- and polysaccharides in food and agricultural products.

BioLC and IonPac are registered trademarks of the Dionex Corporation. Maltrin is a registered trademark of the Grain Processing Corporation. CarboPac is a trademark of the Dionex Corporation.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

In 1983 Rocklin and Pohl showed that carbohydrates could be separated on a high-performance anion-exchange column operated at high pH and detected by pulsed amperometry on a gold working electrode (*1*). Carbohydrates, many of which are uncharged at pH 7, are oxyanions at pH>13 and therefore can be separated by anion-exchange chromatography using NaOH eluents. These eluents were ideally suited for detecting carbohydrates using pulsed amperometry on a gold working electrode. Johnson and Hughes (*2*) had shown that pulsed amperometry was a good technique for the sensitive detection of carbohydrates without sample derivatization. The combination of high-pH anion-exchange separation and pulsed amperometric detection is now known as HPAE-PAD, and is used to analyze for a large variety of carbohydrates in many types of samples (for review see *3-4*).

Koizumi *et al.* (*5*) first described the application of HPAE-PAD to plant-derived linear carbohydrate polymers with high degrees of polymerization (DP). They showed baseline separations of individual glucose polymers up to DP50. This was a major improvement over size-exclusion chromatography, which cannot determine polymers > DP12, and reversed-phase separations, which are limited by the insolubility of large carbohydrate polymers (>DP30) in reversed-phase eluents. Since that publication, separations of amylopectins >DP80 have been achieved (*6*). The high resolution of HPAE-PAD has also been used to analyze a large variety of neutral and charged oligosaccharides (polymers of DP8 or less) and polysaccharides including, amylose/amylopectins (*7*), arabinans (*8*), arabinoxylans (*8*), galactans (*9*), glucans (*5*), glucuronoarabinoxylans (*10*), fructans (*11*), mannans (*12*) oligogalacturonic acids (pectins) (*13*), rhamnogalactans (*14*), xylans (*15*), and xyloglucans (*16*). In this paper I will refer to the oligo- and polysaccharides that can be analyzed by HPAE-PAD simply as oligosaccharides.

Koizumi *et al.* (*7*) later showed that HPAE-PAD could be used to profile the distribution of amylopectins in a variety of agricultural products (e.g., corn, potato, tapioca, etc.). Profiling a given carbohydrate polymer from different sources or from different production lots is now a major HPAE-PAD application. This is perhaps best exemplified by a publication where the fructooligosaccharide content of over 80 fruits, vegetables, and grains was determined (*17*). HPAE-PAD can also be used to follow the distribution of oligosaccharides during a process such as barley malting (*18*) or beer brewing (*19-20*).

While the high resolution of HPAE makes the above applications possible, other applications also rely on the sensitivity of PAD. Orange juice is sometimes adulterated with beet medium invert sugar and illegally sold as pure orange juice. Although the majority of orange juice carbohydrates are mono- and disaccharides, as little as 5% beet sugar adulteration can be detected by an HPAE-PAD analysis of the oligosaccharides in an orange juice sample (*21*). In adulterated samples, this analysis reveals oligosaccharides that are not normally

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found in orange juice. Honey also contains a high percentage of monosaccharides relative to oligosaccharides, but the sensitivity of PAD allows a determination of these oligosaccharides (*22*). The distribution of honey oligosaccharides can suggest the geographical origin of the honey. When refining sucrose from cane sugar, thermal degradation can decrease sucrose yield. Under these conditions one type of sucrose degradation is the formation of oligosaccharides. Although these oligosaccharides are present in very small amounts relative to sucrose, the high capacity of the anion-exchange column and the sensitivity of PAD allow the analyst to monitor the refining process for these degradation products (*23*).

Here I will discuss the principles of, and improvements to, HPAE-PAD technology. The importance of these improvements to the design of HPAE-PAD oligosaccharide determinations is also discussed.

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Materials and Methods

Materials

Sodium hydroxide (50% solution) was obtained from Fisher Scientific (Pittsburgh, PA). Sodium acetate (anhydrous) was purchased from Fluka (Milwaukee, WI). Chicory root inulin was purchased from Sigma Chemical (St. Louis, MO). Maltrin® M040 was obtained from the Grain Processing Corporation (Muscatine, IA).

Equipment

HPAE-PAD was performed on a BioLC® chromatography system consisting of a GP50 gradient pump, an ED50 electrochemical detector with cell, an AS50 autosampler with thermal compartment, and outfitted with either a CarboPac™ PA1 or CarboPac PA-100 column set (guard and analytical) (Dionex, Sunnyvale, CA). The system was controlled and data acquired and processed using PeakNet 6 chromatography software (Dionex). Chromatography eluents were prepared as previously described (*24*).

HPAE

The IonPac® AS6 and its successor the CarboPac® PA1 were used for the initial HPAE oligosaccharide separations. These separations used an eluent with 100 or 150mM NaOH and a gradient of sodium acetate (NaOAc) to as high as 600mM. In general, the larger the oligosaccharide the later it elutes from the column. Koizumi *et al.* (*5*) separated seven glucose polymers that differed in their linkage and showed that for a given DP an oligosaccharide with one linkage would elute at a different time than an oligosaccharide with a different linkage. For example, an α1,6 DP7 glucose oligosaccharide elutes before a β1,3

DP7 glucose oligosaccharide. For amylopectin, a DP*n* oligosaccharide containing one α1,6-linked glucose and (*n*-1) α1,4-linked glucose will elute before a DP*n* oligosaccharide containing only α1,4-linked glucose (*25*).

The CarboPac PA1 resin is composed of surface-sulfonated 10-μM cross-linked polystyrene beads onto which 285-nm cross-linked polystyrene beads, covered with quaternary ammonium anion-exchange groups, are electrostatically bound. This construction yields short diffusion path lengths and therefore high peak efficiencies, resulting in the separation of similar carbohydrates. The CarboPac PA1 resin also allows chromatography using eluents of any pH, most importantly eluents of pH>13. The PA1 was modified to create the CarboPac PA-100, a column designed for better oligosaccharide separations. The PA-100 has smaller beads (8.5-μM coated with 275-nm latex particles) and higher cross-linking. The smaller beads yield shorter diffusion path lengths and less capacity (90% of the PA1) that results in more efficient peaks that can be eluted with less NaOAc. The higher cross-linking allows the column to be used with common reversed-phase solvents (e.g., acetonitrile), which may be important for column cleaning or analyzing solvent-containing samples. A comparison of the PA-100 to the PA1 is shown in Figure 1. An equal amount of a maltodextrin sample, Maltrin M040, separated on both column sets under identical chromatography conditions and detection parameters shows that due to its higher peak efficiency, the PA-100 column (Panel B) separates three additional polymers not separated by the PA1. The higher efficiency of the PA-100 is evidenced by the increased peak heights and narrower peak widths in Panel B compared to Panel A. For example, peak 10 has a height of 0.34 μC and width of 0.33 min in A and 0.45 μC and 0.23 min in B. The lower capacity of the PA-100 is shown by the shorter retention times in B compared to A. Adjusting the gradient conditions for the PA1 column to reflect its higher capacity compared to the PA-100 (i.e. to yield similar retention times) did not result in the separation of any additional peaks.

Since HPAE-PAD was first described, liquid chromatography pumps that are suitable for HPAE-PAD and can deliver curved gradients were commercialized. Despite the availability of such pumps, I was unable to find a published method that used a curved gradient to separate a carbohydrate polymer, although many chromatographers have used multiple-step linear gradients to simulate curved gradients. Figure 2 shows the benefit of curved gradients for oligosaccharide separations. Panels A and B use the same beginning and ending amounts of NaOAc, but the rate of acetate delivery increases in a non-linear fashion in B (i.e., at 30 minutes the column has experienced a higher acetate concentration in A than it has in B). Although both separations resolve about the same number of peaks, the peak spacing is clearly better when using the curved gradient. This allows easier purification of individual peaks and detection of polymers containing single glucose branches.

Published HPAE-PAD separations of oligosaccharides have used either 100 or 150mM NaOH. Koizumi *et al.* (*5*) noted that 150mM increased the solubility of the larger polymers. Figure 3 shows a portion of the HPAE-PAD separations of M040 using the same conditions as in Figure 1B except that 150mM NaOH

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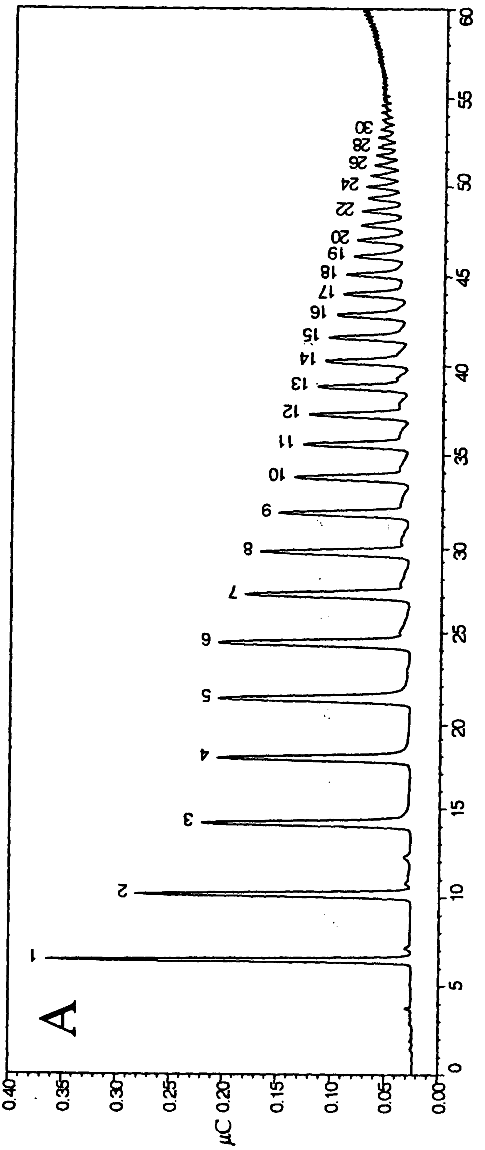

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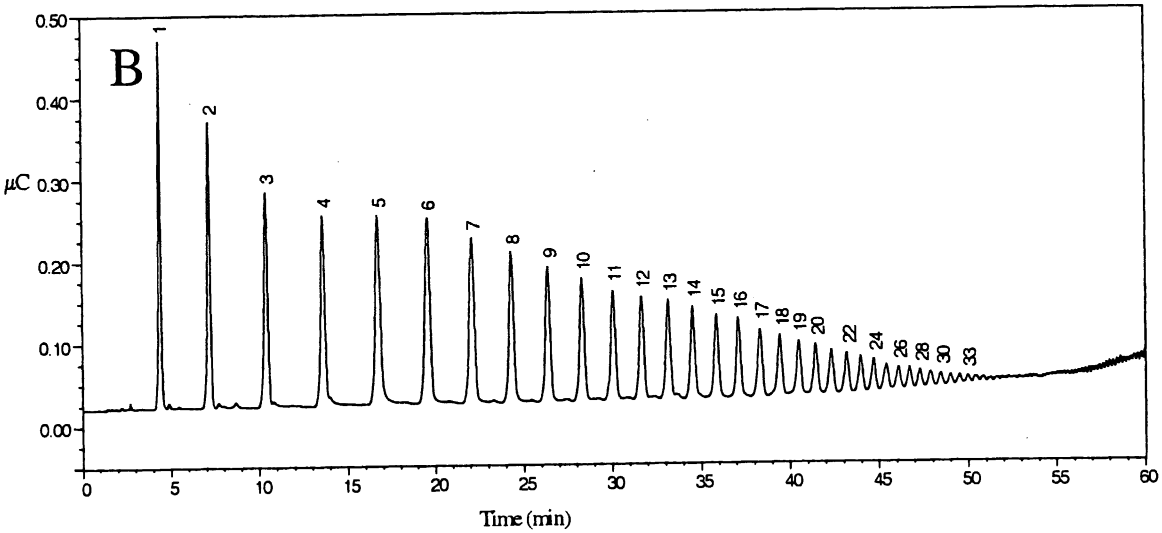

Figure 1. HPAE-PAD separation of Maltrin M040 using a CarboPac PA1 column set (A) and a CarboPac PA-100 column set (B). A 10-μL injection of a 5.2-mg/mL solution of Maltrin M040 was separated with a 50 to 450mM curved (curve 6 in PeakNet 6) NaOAc gradient over 60 min in 100mM NaOH at 1.0 mL/min and a column temperature of 30°C. The system was re-equilibrated at starting conditions for 15 min prior to the next injection. Detection conditions were 0 s 0.1V (v. Ag/AgCl), 0.4 s 0.1V, 0.41 s –2.0V, 0.42 s -2.0V, 0.43 s 0.6V, 0.44 s –0.1V, and 0.5 s –0.1V. Current was integrated between 0.2 and 0.4 s. Maltotriose is peak #2. Glucose is observed in some samples at about 2.2 min (retention time on the PA-100).

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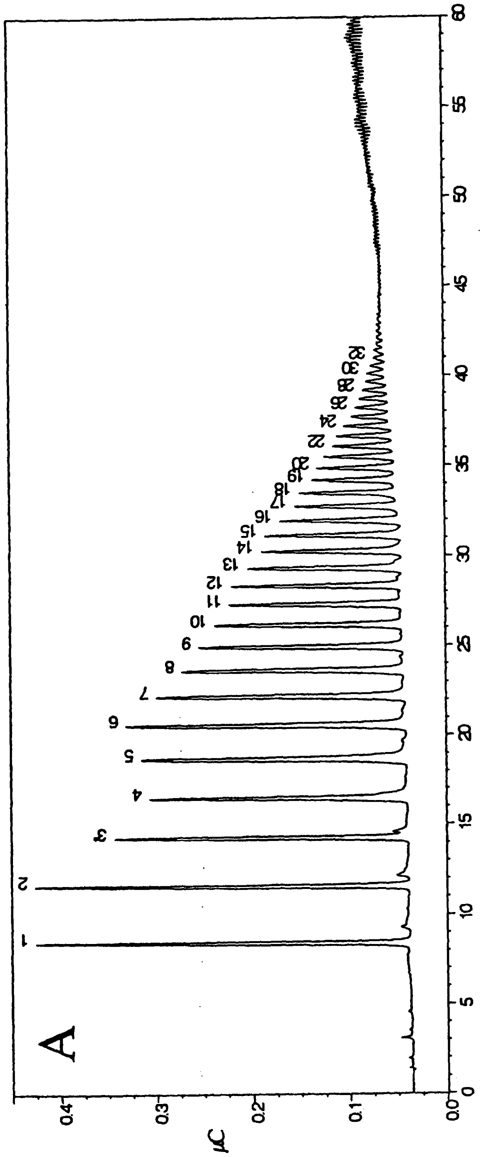

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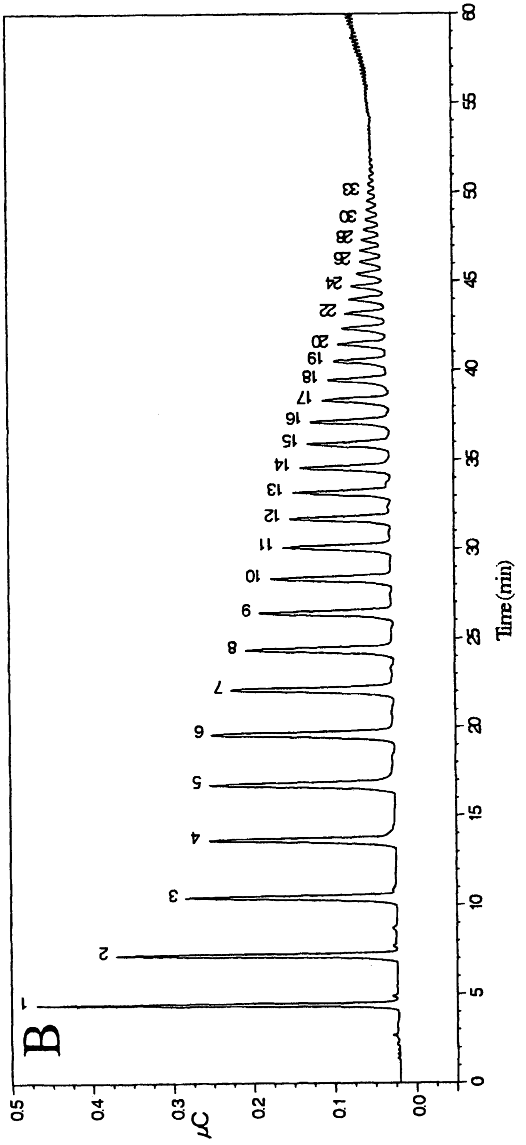

Figure 2. Separation of Maltrin M040 with a linear (A) and a curved (B) gradient. Conditions are the same as Figure 1B except that a linear gradient is used in Panel A.

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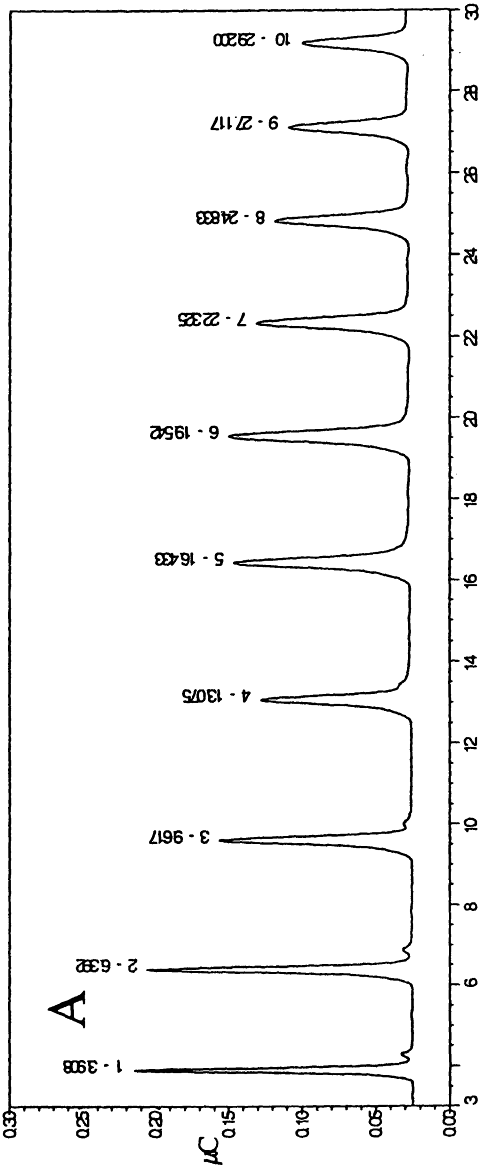

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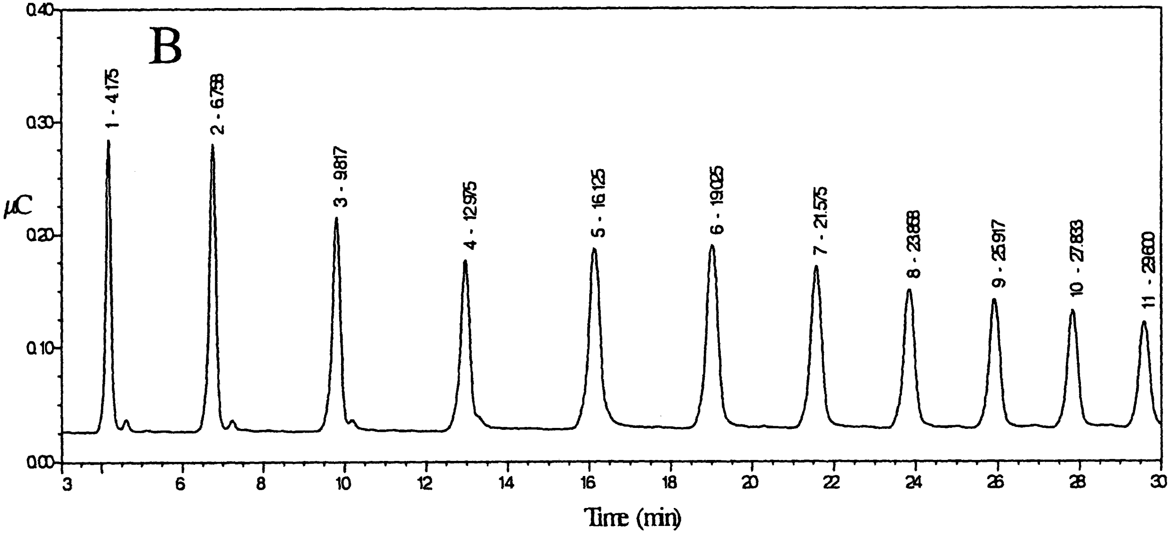

Figure 3. Separation of Maltrin M040 with 150mM NaOH (A) and 100mM NaOH (B). Conditions are the same as Figure 1B except the [NaOH] in A has been changed from 100 to 150mM.

was used in Panel A. Although the eluent in the 150mM NaOH separation has a greater ionic strength at every time point, only peak 1 is eluted earlier. In the portion of the chromatogram depicted in Figure 3, eleven peaks elute with the 100mM NaOH eluent and only ten elute with the 150mM eluent. This is probably due to the increased [NaOH] causing greater hydroxyl group ionization. Increased ionization results in greater retention that is not balanced by the eluting power of the increased ionic strength. This has also been observed for branched oligosaccharides (*26*). For M040, the 150mM eluent did not improve the separation, which is probably a result of the sample being readily soluble in water. One hundred fifty millimolar NaOH is beneficial for less soluble samples and samples containing larger polymers. These are undoubtedly the reasons that 150mM NaOH was chosen for the majority of the published separations. If the analytical goal is to purify individual saccharides for another analysis (e.g., mass spectrometry), using 100mM NaOH will reduce the amount of salt that may need to be removed from the purified sample.

The results from Figures 1, 2, and 3 suggest that HPAE-PAD oligosaccharide separations should be developed using the CarboPac PA-100 column, a curved gradient, and either 100 or 150mM NaOH depending on the nature of the sample and the analytical goals of the analysis. These principles were used to develop an HPAE-PAD separation of chicory inulin, a fructan. After using a broad NaOAc gradient (20-800mM) to determine where the first and last major peaks eluted, a gradient of 150-550mM was chosen. An evaluation of curved gradients available in PeakNet 6 found once again that curve 6 yielded the best separation. These conditions were used to evaluate two lots of chicory inulin that were purchased over a decade apart (Figure 4). By dividing the peak height of each peak in Panel A by the peak height of the same peak in Panel B, I found that the lot in A had a greater percentage of the larger saccharides and a smaller percentage of the smaller oligosaccharides. Figure 4 is an example of how the high resolution of HPAE and its ability to determine large polymers has made HPAE-PAD ideal for evaluating product lots and carbohydrate polymers from different foods or agricultural sources.

Although the majority of HPAE separations have used NaOH and NaOAc, other eluents that are compatible with PAD have been used. Wong and Jane (*27*) showed that sodium nitrate could be used instead of NaOAc for oligosaccharide separations. They compared the two eluents for separating an amylopectin sample. Because nitrate is a stronger eluent than acetate, lower eluent strengths can be used. Using a four-step gradient for acetate that began with 125mM and ended with 400mM and a five-step gradient for nitrate that began with 30mM and ended with 125mM (both eluents had 150mM NaOH) they found that the nitrate method could determine up to DP66 while the acetate could only determine up to DP58. This was attributed to greater PAD sensitivity in the nitrate eluent. The authors also concluded that the nitrate eluent system was better for separating linear from branched isomers.

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Polygalacturonic acid, the carbohydrate backbone of pectin, is negatively charged at neutral pH; and therefore, does not require alkaline eluents to bind to the CarboPac columns. Some of the galacturonic acids in pectin contain methyl esters, the number of which affects pectin function, including its ability to gel. Because methyl esters are hydrolyzed at high pH, polygalacturonic acid is separated with eluents near neutral pH and NaOH is added post-column to detect the sugars by pulsed amperometry. Hotchkiss and Hicks found that NaOAc eluents (pH 5) could separate oligogalacturonic acids < DP20, and stated that the larger polymers were not soluble in the acetate eluent (*28*). To separate larger polymers (up to DP50), they used potassium oxalate (pH 6) eluents. The CarboPac PA1 was reported to be superior to the PA100 for separating polygalacturonic acid polymers with methyl esters (*29*). Other detection possibilities for HPAE pectin separations are UV absorbance after prior derivatization with 2-aminopyridine (*30*) and visible absorbance (525 nm) (*31*), after reacting the column effluent with potassium permanganate.

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PAD

Since PAD of carbohydrates was first described there have been improvements to the series of potentials applied to the working electrode (the waveform). Prior to 1997 these waveforms all applied three potentials to the gold working electrode. The first potential was used to oxidize and detect the carbohydrate, the second potential created a gold oxide surface to clean the electrode surface, and the third potential was used to restore the gold surface. Although these waveforms yielded good short-term peak area reproducibility, there was a gradual loss of the gold surface that caused a long-term loss of peak areas. The discovery that strong reductive potentials could be used to effectively clean the working electrode without loss of the gold surface (*32*) led to the development of a four-potential waveform that yields good long-term peak area reproducibility (*33-34*). The first potential is used to oxidize and detect the carbohydrate, the second reductive potential (-2.0V v. Ag/AgCl) is used to clean the electrode surface, the third short potential reactivates the electrode by transiently forming an oxide surface, and it is removed with the fourth potential to reestablish the gold surface. All the separations shown here use this four-potential waveform and all new HPAE-PAD methods should be developed using this waveform. This is especially important for applications in which production lots of an oligosaccharide product must be compared over a long period of time. Although a strong alkaline pH is necessary for sensitive carbohydrate detection, it was recently shown that PAD could detect oligogalacturonic acids at pH 7.5 when preparative amounts (e.g., 50 mg, approximately 1000X an analytical separation) are separated (*35*).

There are a few published studies of the relative electrochemical responses of neutral oligosaccharides (*5,7,25,36*). Although these studies have been conducted with different waveforms and different eluent conditions they all reached the same conclusions. Using amylopectins, Koizumi *et al.* (*5,7*) found that response increased with increasing DP and this increase could be normalized to an increase per hydroxyl group. This is consistent with two later

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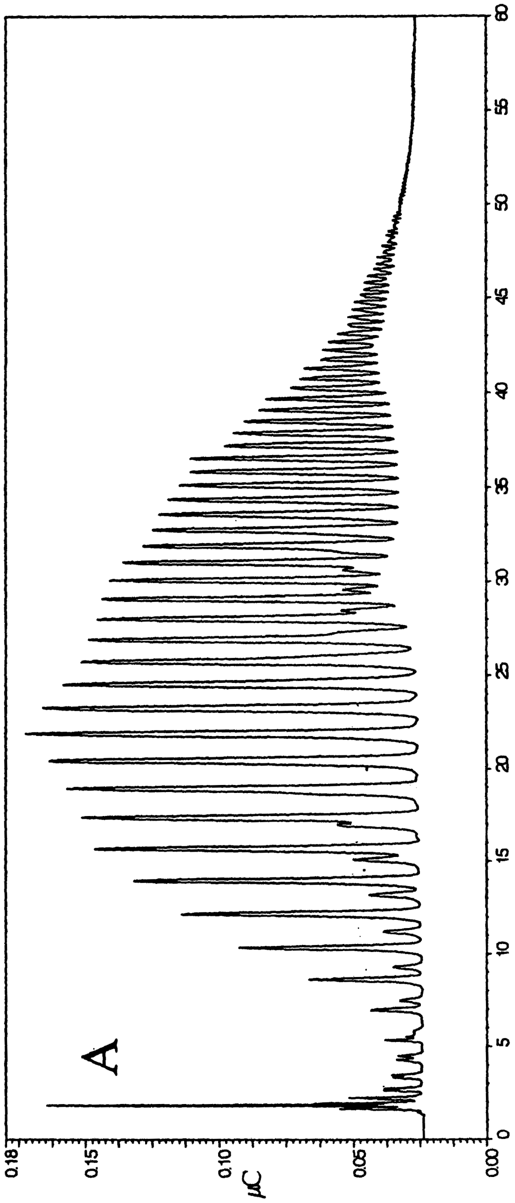

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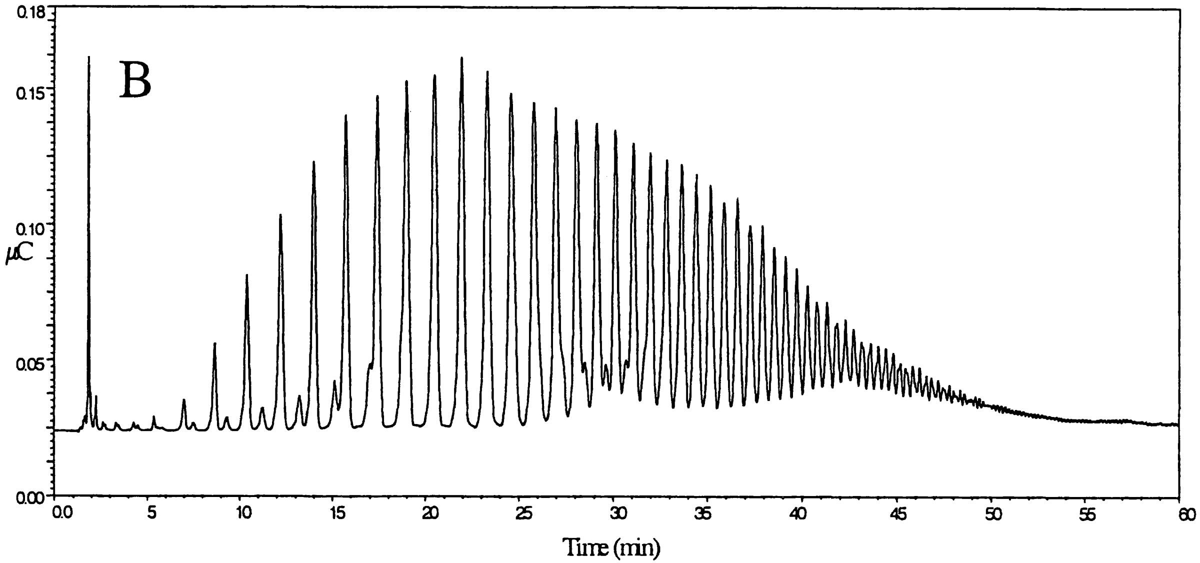

Figure 4. HPAE-PAD analysis of two lots of chicory inulin. The samples were dissolved in 100mM NaOH. A 10-μL injection of a 5-mg/mL solution of chicory inulin was separated with a 150 to 550 mM curved (curve 6) NaOAc gradient in 100 mM NaOH. Other conditions are the same as Figure 1.

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publications that showed that as amylopectin DP increased there was a linear increase in response per micromole injected and a decrease in response per microgram injected (*25,36*). Although the response increases per micromole injected as DP increases, the profile gives an approximate ratio of the molar distribution of the carbohydrate polymer. For example, amylopectins of DP6 and DP17 have responses of 0.74 and 1.65 when DP9 is set to 1.0 (*7*). Hotchkiss and Hicks studied the PAD response for oligogalacturonic acids (*28*). They reported that response decreased with DP, unlike neutral oligosaccharides. This is believed to be due to the negatively charged carboxylate groups inhibiting contact with the working electrode surface. They also noted that electrochemical response for a given oligosaccharide varied with eluent conditions.

Conclusions

Since its introduction in 1983, .HPAE-PAD has proven to be a valuable technique for scientists studying processes to which oligosaccharides are important (e.g., sucrose refining), purifying oligosaccharides, assaying oligosaccharide products and products containing oligosaccharides, and authenticating food and agricultural products by oligosaccharide composition. This is a result of the high-resolution separations of HPAE and the sensitive detection of PAD. Here I reviewed how the basic separation and detection principles of HPAE-PAD have been applied to developing oligosaccharide analysis methods. New HPAE-PAD oligosaccharide methods should use the CarboPac PA-100 column, 100 or 150mM NaOH with a non-linear NaOAc gradient, and a four-potential waveform that uses reductive electrode cleaning.

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Chapter 3

The Analysis of Oligosaccharides by Mass Spectrometry

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Over the last decade, great improvements have been made in the use of mass spectrometry to analyze large molecules. Two ionization techniques, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) permit the determination of molecular weights of macromolecules in excess of 100,000 Daltons. The MALDI-time of flight mass spectrometric analysis of polysaccharide chains, including dextrans and debranched amylopectin was performed employing 2,5-dihydroxybenzoic acid as the sample matrix. Analysis of debranched amylopectin from rice by MALDI-TOF showed adducts of sodiated maltodextrins containing from 3 to 45 glucose residues. A typical mass spectrum contained a series of peaks differentiated by a mass of 162 Daltons, indicative of a single glucose monomer. Relative intensities increased to a maximum of 9 or 10 residues depending upon the sample. Thereafter, a gradual decrease in peak intensity was observed with the addition of each successive glucose unit to a maximum of 45 monomers.

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Introduction

The primary determinant for classifying rice based on its physical properties is the amylose/amylopectin ratio. However, there are rice cultivars that have low amylose content which possess gelatinization temperatures that range from low to high. That is, amylose content is at best, crudely associated with gelatinization temperature. Instead, the fine structure of amylopectin impacts aspects of starch functionality. The functional characteristics, in milled rice, reportedly controlled by the relative distributions in amylopectin chain length are gelatinization temperature, cooked kernel texture and noodle quality (*1-5*). Debranching amylopectin results in a mixture of maltodextrins varying only in the degree of polymerization. Oligosaccharides dominate this mixture, and the distribution of debranched amylopectin can be related to the functional properties of the starch. Much of this work has been performed using gel permeation chromatography (GPC) to separate debranched amylopectin into partially separated groups of varying chain lengths. For rice, a trimodal distribution is generally found which consists of short (<25), medium (25-65) and long (>65) oligomer chains (*6-7*). The longer chains are those that appear to contribute firmness to both cooked rice and rice-based foods (*4, 8*).

Although much work has been accomplished using GPC, there is a need for greater resolution for accurate description of the polymeric distribution of the debranched amylopectin. There are no less than three promising techniques which may provide this resolution; HPLC, capillary electrophoresis (CE), and matrix assisted laser desorption/ionization - time of flight mass spectrometry (MALDI-TOF). High performance anion-exchange chromatography with pulsed amperometric detection can resolve debranched amylopectin with degrees of polymerization between 60 and 85 glucose units (*9-10*). For CE, separation of amylopectin chains up to 100 glucose units has been achieved (*5,11*). Whereas, GPC and SE-HPLC separate native and debranched amylopectin into groups based on their hydrodynamic volume (*12*), CE separates the chains based on charge, and MALDI-TOF separates the chains based on molecular weight. In initial work, Hillencamp *et. al.* employed MALDI-TOF to observe various dextrans (*13*).

An additional challenge arises from the need to isolate oligosaccharides from the complex natural matrices in which they are often found. A hyphenated technique which combines chromatographic separation prior to analysis, enhances the results with respect to the individual oligosaccharides (*14-15*). An additional approach has been the derivatization of the carbohydrates to enhance sensitivity for both HPLC and MS detection (*14-16*). The object of this chapter is to give a brief history of the analysis of oligosaccharides by MALDI-TOF and to apply the method for the characterization of the polysaccharides obtained by debranching the amylopectin extracted from rice.

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Oligosaccharides and Mass Spectrometry

Analysis of oligosaccharides by mass spectrometry was initially limited to derivitized compounds. Because most mass spectrometers were limited in their upper mass range, this effectively limited the analysis to mono- through tetrasaccharides on all but the most advanced instruments. With the introduction of plasma desorption and fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS) in the early 1980's the door was opened for the analysis of large biomolecules (*17*). For the first time, large molecules could be ionized and vaporized in the gas phase without undergoing severe degradation by the process. However, the techniques proved effective for analyzing only mono-, di- and trisaccharides.

Over the last decade, great improvements have been made in the use of mass spectrometry to analyze large molecules, and in particular biomolecules. Electrospray was introduced and coupled with HPLC for on-line analysis (*18-19*). Electrospray ionization imparts multiple charges onto the large biomolecules. Because mass spectrometers measure the mass-to-charge ratio, a compound with a nominal mass of 1,000 Da with two charges would produce a signal at 500 *m/z*. The multiply-charged analytes can now be measured in mass spectrometers that would otherwise have insufficient mass range to analyze these compounds if based on a singly-charged ion. Electrospray has proven invaluable for the analysis of proteins and peptides. As a general rule, one unit of charge is added for every 800-1000 Da in mass with most ESI spectra of large molecules falling between 800-2000 m/z. Unfortunately, due to the high polarity of oligosaccharides, only the mono-, di- and trisaccharides are normally observed. However, when bound to less polar compounds, as in the case of glycolipids and glycoproteins, glycosyl residues and linkages can be determined.

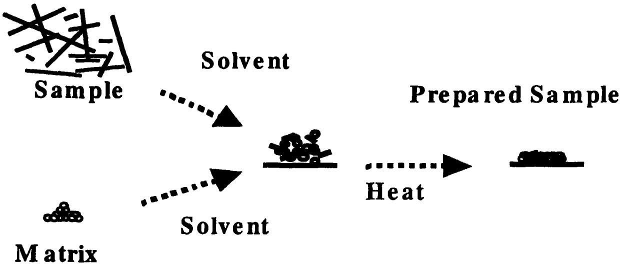

Figure 1. Sample preparation of debranched amylopectin by co-crystallization with a suitable matrix for analysis by MALDI-TOF.

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MALDI-TOF mass spectrometry was originally introduced in the late 1980's (*20*) as a new ionization technique for biomolecules – particularly peptides and proteins (*21-22*). In this technique, sample molecules and matrix molecules are dissolved in solvents and co-deposited on a laser target (Figure 1). The matrix is selected to absorb at the laser wavelength and to promote the ionization of the sample molecules. The sample is then gently heated to evaporate the solvent, resulting in the crystallization of the sample within the matrix.

Once crystallized, the sample target is placed in the vacuum chamber for analysis. A laser pulse is fired at the target (Figure 2). The actual desorption/ ionization event is not completely understood at this time. The laser pulse also triggers the beginning of the time-of-flight analysis of the ions generated. The singly charged ions are accelerated down a flight tube to an electron multiplier where their arrival time and intensity are recorded. This information is then converted to a mass spectrum based upon the relationship between mass and velocity. Although the majority of applications of MALDI-TOF are related to protein and peptide research, investigations of other macromolecules are becoming more prevalent. Synthetic polymers are now routinely characterized by MALDI-TOF (*23-25*). For starch analysis, DHB was found to serve as a good matrix for the analysis of debranched amylopectin from corn and barley (*26*). Debranched amylopectin from waxy maize and potato have been analyzed using MALDI-TOF where up to 65 glucose residues were observed (*27*). Kuhn *et al*, in a comparitive study analyzing oligosaccharides, reported observations of up to 45 glucose residues using HPAEC-PAD and up to 60 using MALDI-TOF (*28*).

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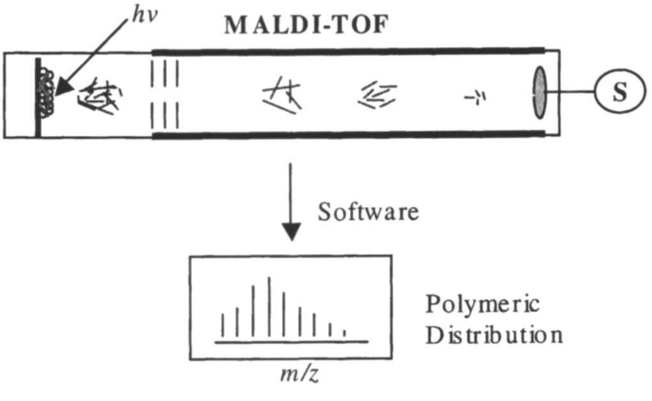

Figure 2. Block diagram of MALDI-TOF.

The MALDI-TOF spectrum generally shows a peak at every repeating unit of the polymer, and the distribution of the intensities of these peaks is used to calculate the polymer characteristics. Information that can be gained from a mass spectrum of a polymer, includes the average molecular weight (MW), average mass number (Mn) and polydispersity index (PD). The mass resolution determines whether each repeating unit may be observed and is increasingly important as mass increases. Fine information including determination of end-groups can also be extracted in some cases (*24*).

Experimental

Source of Materials

Starch was separated from a milled sample of the U.S. rice cultivar 'Cypress' using the alkaline method reported by Yang et al. (*29*). The rice starch was then defatted overnight using methanol. The starch was solubilized in dimethylsulfoxide (DMSO) and debranched using isoamylase (*30*). Dextran (G_{1-23}) was obtained from Oxford Glycosciences (Oxford, U.K.). Samples of maltotriose through maltoheptaose were obtained from Sigma-Aldrich (St. Louis, MO).

Matrix and Solvent Optimization

The solulibization of starch is problematic. Oligosaccharides readily dissolve in water while longer chains require the use of organic solvents such as methanol and/or DMSO. Table I lists the various matrix compounds and our abbreviations which were employed as matrices in this study. These compounds were purchased at Sigma-Aldrich.

Table I. Common Matrix Compounds

Name	Abbreviation
Ferulic Acid	FA
2,5-dihydroxy benzoic acid	DHB
α -cyano-4-hydroxy cinnamic acid	α-CN
2,5-dimethoxy cinammic acid	DMC
[2-(4-hydroxyphenylazo)]-benzoic acid	HABA

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MALDI-TOF Analysis

Analytes were dissolved in either water, methanol or DMSO at a concentration of 1 mg/ml. The matrix, normally DHB, was dissolved until saturated in the same solvent as the analyte. If the solvent was water, methanol was added to increase the solubility of the organic matrix molecule. An aliquot consisting of 0.5 µl of the matrix solution was deposited onto the target followed by 0.5 µl of the sample. The target was then placed on a heating plate at about 50°C for ~ 5 minutes to remove the solvent. A Finnigan LaserMat 2000 equipped with a nitrogen laser was used for this study. Intensity was manually selected to just above detection of positive ions and then 100 laser shots were collected and averaged. For optimization of high mass spectra, low mass deflectors were employed for the collection of spectra above 1000 Da.

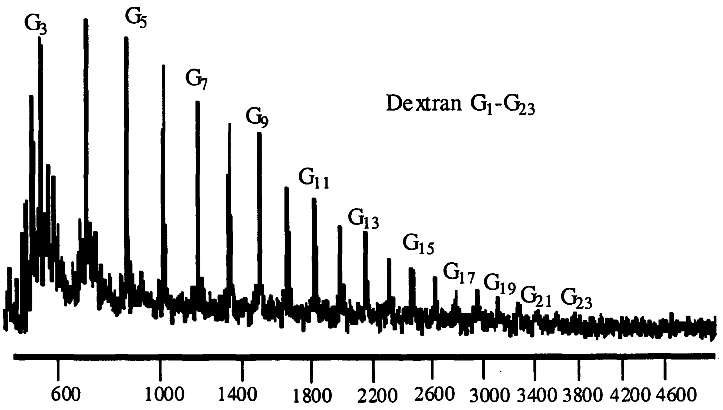

Figure 3. Mass spectrum of a mixture of dextrans (glucose α 1-6)$_n$ by MALDI.

Results and Discussion

Optimization of the MALDI parameters was determined by analyzing a debranched amylopectin sample for optimal signal while varying the matrix, solvent, and the use of high-mass deflectors. The matrices employed are listed

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in Table I. Water, methanol and DMSO were employed as solvents for both the matrix and the sample. The best signal-to-noise ratio was obtained using methanol as a solvent, DHB as the matrix and the having the deflectors on. The best mass resolution was obtained using DMSO or methanol and ferulic acid or DHB.

A mass spectrum of a commercial mixture of dextrans was analyzed by MALDI-TOF (Figure 3). The very low molecular weight region (< 500 Da) was dominated by the presence of the matrix peaks and is not shown. The spectrum shows the adducts of the molecular ions resulting from the addition of ubiquitous sodium. Although neither sodium or potassium are added to the matrix, sufficient quantities are present to produce the adducts of the oligosaccharides. Successive peaks from 3 to 23 glucose units were observed. The largest quantity of the dextrans observed was the sodiated G_4 peak. Although the monomer and the dimer were present in the sample, they were not observed as either the H^+ or Na^+ adducts. The trimer was observed as both the protonated species and the Na^+ species. To a lesser extent, the H^+ form of the tetramer was also present, with the Na^+ species dominant in the remainder of the series. In comparison, a GPC of the standard mixture supplied by the manufacturer showed glucose as the most abundant compound, and the concentration decreased with the addition of each successive glucose unit until only a trace of the G_{23} compound was observed. With the exception of G_1-G_3 this intensity pattern is observed in the MALDI-TOF spectrum.

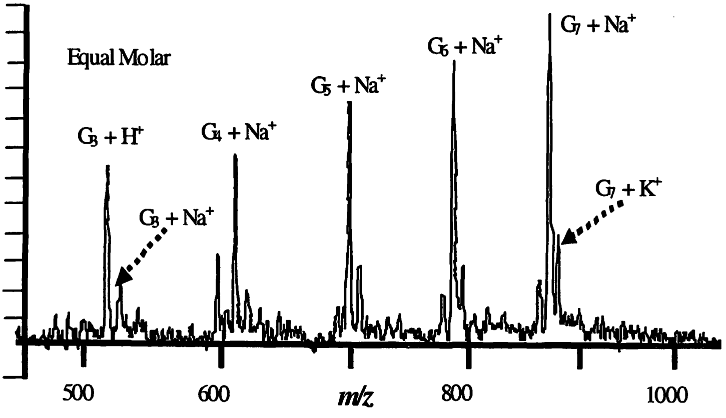

Figure 4. MALDI spectrum of an equimolar mixture of malt glucose units.

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In order to determine the relative response of the oligosaccharides, an equimolar mixture consisting of 0.1 M each, of maltotriose through maltoheptose (Glucose α1-4)$_{3-7}$ was prepared for MALDI analysis employing DHB as the matrix and water as the solvent. Figure 4 shows the mass spectrum obtained by summing the data from 100 laser shots. Relative peak intensities increase with the addition of each successive glucose unit. This phenomena has been observed previously (*31*) and in general there is an increase up to 1,000 Da (G$_8$) after which the ionization and matrix effects are not significantly different between successive oligosaccharides. Comparing the intensities from a TOF and a sector mass spectrometer, the cause was determined to be a temporary saturation of the detector resulting from large number of matrix ions. Of particular interest is the relative amount of the H^+ form to the Na^+ form of the molecular adducts of maltotriose. In the tetrasaccharide and higher oligosaccharides, the Na^+ form dominates. The K^+ form is also observed in the tetra and higher olicosacchardies, but not in the trisaccharide.

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MALDI-time of flight (TOF) mass spectrometry of debranched amylopectin was performed by dissolving the sample in DMSO and employing DHB as the sample matrix. Low mass deflectors were employed to prevent the saturation of the detector and enhance the high mass end of the spectrum. Analysis of debranched amylopectin from rice by MALDI-TOF showed molecular adducts of oligosaccharides and sodium containing from 5 to 45 glucose residues (Figures 5A & 5B). The series of peaks were differentiated by a mass of 162 Daltons, the equivalent of a single glucose residue. Relative intensities increased to a maximum of 9 or 10 glucose residues depending upon the sample. Thereafter, a gradual decrease in peak intensity was observed with the addition of each successive glucose residue. In spectra of debranched amylopectin obtained without the use of low mass deflectors (data not shown), maltotriose and maltotetrose were observed as the sodium adducts. Due to the limitations of the technique it is not known if glucose or maltose were present. In a starch sample where the amylose had not been separated, the response of the peak intensities was lower as would be expected do to the decrease in relative concentration. Using HPAEC and CE, other researchers have reported similar chain length profiles for various rice cultivars (*5, 10*). A maximum is also found using GPC at ~10 to 15 glucose residues (*6-7)* and similar to our observations.

Variation in the distribution of amylopectin chains between starch sources such as corn, barley, or rice can be differentiated from one another based upon their mass spectra (27-28). The changes in the distribution of the branches can also be discerned from one rice sample to another. It is this variation that relates back to the fine structure of amylopectin in rice samples, and which is believed to affect the cooking properties of rice. MALDI-TOF provides an accurate snapshot of all the rice debranched glucose polymers except for glucose, maltose and the very longest chains. Using MALDI-TOF, dozens of samples could be screened per day making this method practical for screening for the cooking properties of rice by industrial food processors.

Although not utilized in this research, MALDI-TOF can be used for elucidating the structure of carbohydrate moieties of biologically important macromolecules, i.e. glycoconjugates. Limited fragmentation does occur under MALDI and can be enhanced to aid in identification (*32*). Analogous to mass spectra produced by electron impact ionization, fragmentation patterns can be

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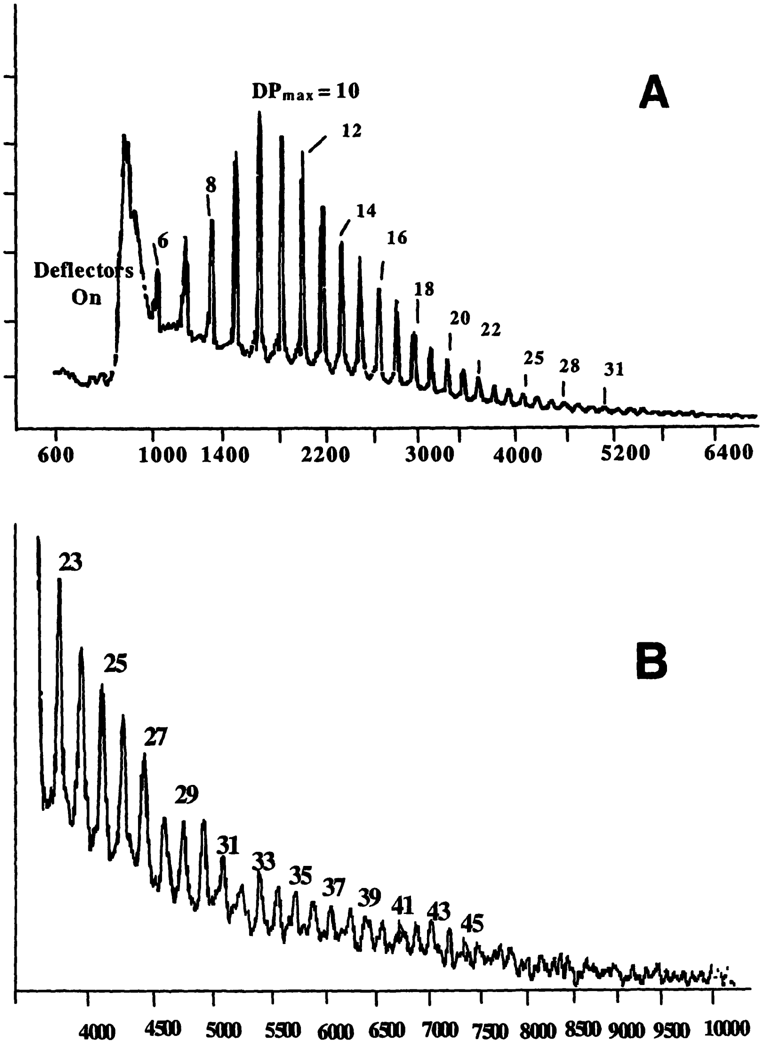

Figure 5. MALDI-TOF mass spectrum of debranched amylopectin. A). Total mass spectrum B). Detailed veiw of the high mass region.

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used to differentiate between different hexose isomers such as glucose, fructose, and mannose. The sequencing of oligosaccharides consisting of different hexose isomers has been reported (*33*), but is by no means a routine technique. Combining techniques such as derivitization, enzyme degradation, and instrumental techniques such as post source decay or time-lag focusing, fragmentation patterns yield not only isomeric information, but information on branching as well (*34*). MALDI-TOF will continue to play a key role in the structural elucidation of biological macromolecules.

Conclusions

It has been shown that the MALDI-TOF technique has the ability to form cationic adducts of crystallized mixtures of oligosaccharides with H^+, Na^+ or K^+. The technique can completely resolve mixtures of oligosaccharides based upon their molecular weight. However, mono- and disaccharides were not ionized and consequently were not observed in a mass spectrum consisting of a mixture of dextrans. A bias was observed in the ionization process of an equimolar mixture of maltosaccharides from G_3-G_7. The observed peak intensity increased with the addition of each successive glucose unit. This is consistent as reported in the literature where intensity increases up to a mass of 1000 Da. For an equimolar mixture and then levels off. Separation of the amylose is not required before analysis but affects the sensitivity of the process. However, the major advantage of MALDI-TOF over GPC is that all the data for all the glucose polymers will be collected in less than 1 min compared to 30-40 min for GPC. This advantage should greatly increase sample throughput.

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Chapter 4

Potential for the Development of Prebiotic Oligosaccharides from Biomass

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Waste biomass contains a multitude of complex carbohydrate molecules. These carbohydrates can be considered as a resource for the development of novel prebiotic oligosaccharides which may have better functionality than those currently established on the market. Enhanced persistence of the prebiotic effect along the colon, antipathogen effects, and more closely targeted prebiotics, might all be possible starting from plant polysaccharides. Of particular interest for the development of novel prebiotics are oligosaccharides from arabinoxylans and pectins. Oligosaccharides derived from the breakdown of both classes have received increased research attention recently. The development of prebiotics based upon biomass will demand the development of new manufacturing technologies.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Prebiotic Oligosaccharides

At the present time the only molecules known to act as prebiotics are carbohydrates (*1-2*), in particular, non-digestible oligosaccharides. The first requirement of a prebiotic is that it is not metabolized in the human small intestine and passes to the colon (the main site of microbial activity in the body) quantitatively. The key defining feature of prebiotics, however, is not their non-digestibility, nor the fact that they are fermented in the colon, but that this fermentation is selective towards certain, desirable, members of the colonic microflora. The usual targets for prebiotic fermentation are the indigenous probiotic bacterial genera, *Bifidobacterium* and *Lactobacillus* (*1*).

There are several prebiotics in use around the world with Japan being the leader in prebiotic food development (*3-5*). In Europe and the USA there are three prebiotic oligosaccharides on the market: the fructans, inulin and fructooligosaccharides (FOS) and galactooligosaccharides (GOS). Lactulose also displays prebiotic behavior at sub-laxative doses (*6*) but is best known as a laxative and is not used in food manufacture at the present time. The range of approved prebiotic oligosaccharides in Japan is more extensive and it is likely that these will ultimately be used on a global scale. These can, therefore, be considered as "emerging prebiotics" (Table I).

Table I. Emerging Prebiotics on the Global Market

Emerging Prebiotics
Isomaltooligosaccharides (IMO)
Soybean oligosaccharides (SOS)
Lactosucrose (LS)
Gentiooligosaccharides (GEOS)
Xylooligosaccharides (XOS)

These emerging prebiotics have generally not yet been tested as rigorously as the established prebiotics and we have an incomplete picture of their fermentation and technological properties. It is possible that the molecules might have better nutritional properties and more desirable properties to food processors than the established prebiotics.

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Novel Prebiotics

Most of the prebiotics in industrial use are naturally occurring oligosaccharides or are manufactured by enzymatic transfer reactions utilizing cheap raw materials such as sucrose and/or lactose (*5*).

Despite the range of oligosaccharides on the global market, it is likely that other oligosaccharides may have more desirable properties than the existing molecules. Several desirable attributes that a prebiotic might possess are presented in Table II.

Table II. Design Parameters for Enhanced Activity Prebiotics

Desirable attribute in prebiotic	*Properties of oligosaccharides*
Active at low dosage	Highly selectively and efficiently metabolized by *Bifidobacterium* and/or *Lactobacillus* sp.
Lack of side effects	Highly selectively metabolized by "beneficial" bacteria but not by gas producers, putrefactive organisms, etc.
Persistence through the colon	High molecular weight
Varying viscosity	Available in different molecular weights and linkages
Good storage and processing stability	Possess 1-6 linkages and pyranosyl sugar rings
Fine control of microflora modulation	Selectively metabolized by restricted species
Varying sweetness	Varying monosaccharide composition

Although it will very likely be impossible to achieve all of these attributes in a single oligosaccharide, these attributes can reasonably be considered as targets when developing novel prebiotics. Some of these attributes will be considered in more detail in the following sections.

Persistence of the Prebiotic Effect to Distal Regions of the Colon

Many common diseases of the human large bowel arise in the distal colon, particularly colon cancer (*7*). Prebiotics have been postulated to be protective against the development of colon cancer (*8-12*). If prebiotics are to have any protective value, however, they must be selectively fermented in the distal colon.

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One way of achieving this might be control of molecular weight. Most prebiotic oligosaccharides are of low molecular weight, the exception being inulin. Inulin, being a low-molecular weight polysaccharide, is consequently more slowly fermented in the gut than other prebiotics and reaches more distal regions of the colon. Recent product development in this area has led to industrial forms of inulin/FOS mixtures with controlled chain length distributions ("Synergie II" manufactured by Orafti, Tienen, Belgium), which should persist further along the colon.

As plant-derived inulin is intrinsically limited in its DP, plant cell wall polysaccharides might be attractive starting materials for prebiotic development. Such polysaccharides are known to be dietary fibers (*13*) but are not selectively fermented in the colon and are not prebiotics. It is likely that carbohydrates must be of relatively low molecular weight to be selectively fermented as it is thought that microbial cell wall-associated exoglycosidases are responsible for metabolism of prebiotics (*14*). An increase in the selectivity of fermentation upon hydrolysis of a polysaccharide has been reported (*15*). Development of a persistent prebiotic might thus prove to be a compromise between persistence and prebiotic activity.

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Anti-adhesive Activities Against Toxins

The idea of combining prebiotic properties with anti-adhesive activities is currently under investigation. This would add major functionality to the approach of altering gut pathogenesis. Many microbial toxins bind to gut epithelial cells, utilizing monosaccharides or short oligosaccharide sequences as receptors. It is, in principle, possible to block such interactions with soluble oligosaccharides (*16*). Of particular relevance is the binding of *E. coli* verocytotoxins to galactooligosaccharides. The cellular receptors for this toxins are oligosaccharides containing the α–D-Gal*p*(1→4)-D-Gal epitope. The backbone repeating unit of the smooth regions of pectins contains the α–D-Gal*p*A(1→4)-D-GalA sequence, which is a structural mimic of the receptor. Preliminary studies have shown that pectins and oligosaccharides derived from them by hydrolysis can inhibit cytotoxicity caused by verocytotoxins (*17*).

Targeted Prebiotics

The current definition of a prebiotic is an oligosaccharide that is selectively fermented by bifidobacteria and lactobacilli (*1*). Virtually all of the experimental data on the prebiotic properties of oligosaccharides describes the microflora changes at the genus level. This is due to the difficulties in characterizing the

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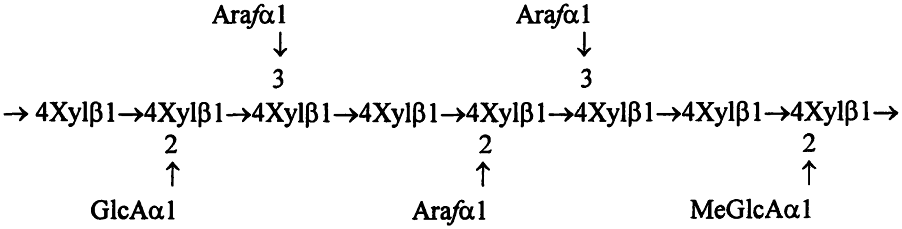
complex colonic ecosystem and of speciation of colonic bacteria using selective media and biochemical tests. With the advent of new molecular techniques of microbial characterization (*18*), however, it is now possible to characterize microflora changes at the species level with a high degree of confidence. Given such capabilities it would be desirable to develop prebiotics targeted at particular species of *Bifidobacterium* and *Lactobacillus*. Such targeted prebiotics would form the basis of a new range of synbiotic products consisting of a probiotic with proven properties and an oligosaccharide targeted at that particular species.

The development of targeted prebiotics might be achieved by screening a wide variety of carbohydrate structures. In this context, plant cell wall polysaccharides are attractive resources with which to enhance the structural diversity of candidate prebiotics.

Biomass as a Source of Prebiotics

Plant cell walls contain a rich diversity of monosaccharide residues and structures. Whilst there has been much interest in the dietary fiber properties of cell wall polysaccharides (*13, 19-20*) there have been very few attempts to date to manufacture prebiotics using plant biomass with the one exception of the Japanese xylooligosaccharides (XOS). Xylans are complex polysaccharides; a generic structure of glucuronoarabinoxylan is given in Figure 1.



$$\rightarrow 4\text{Xyl}\beta 1\rightarrow 4\underset{\underset{\text{GlcA}\alpha 1}{\uparrow}}{\underset{2}{\text{Xyl}}}\beta 1\rightarrow 4\overset{\overset{\text{Ara}f\alpha 1}{\downarrow}}{\overset{3}{\text{Xyl}}}\beta 1\rightarrow 4\text{Xyl}\beta 1\rightarrow 4\underset{\underset{\text{Ara}f\alpha 1}{\uparrow}}{\underset{2}{\text{Xyl}}}\beta 1\rightarrow 4\overset{\overset{\text{Ara}f\alpha 1}{\downarrow}}{\overset{3}{\text{Xyl}}}\beta 1\rightarrow 4\text{Xyl}\beta 1\rightarrow 4\underset{\underset{\text{MeGlcA}\alpha 1}{\uparrow}}{\underset{2}{\text{Xyl}}}\beta 1\rightarrow$$

Figure 1. Generic structure of plant glucuronoarabinoxylans. Xyl: D-xylose, Araf: L-arabinofuranose, GlcA: D-glucuronic acid, MeGlcA: 4-O-methyl-D-glucuronic acid.

XOS are made by enzymatic hydrolysis of corn cob xylan (see below) and are a successful prebiotic product in Japan. Their acid stability enables them to find application in acidic products with a long shelf-life requirement such as carbonated drinks (*4-5*).

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The commercial product is principally xylobiose with some tri- and tetrasaccharide components, but there is potential for development of varying xylooligosaccharide structures by the use of a different xylanase.

It is, perhaps, surprising that cello-oligosaccharides have not been investigated for their prebiotic properties given the interest in saccharification of cellulose to make glucose. In addition, cellobiose has the interesting property of attenuating the virulence of *Listeria monocytogenes* (*21*).

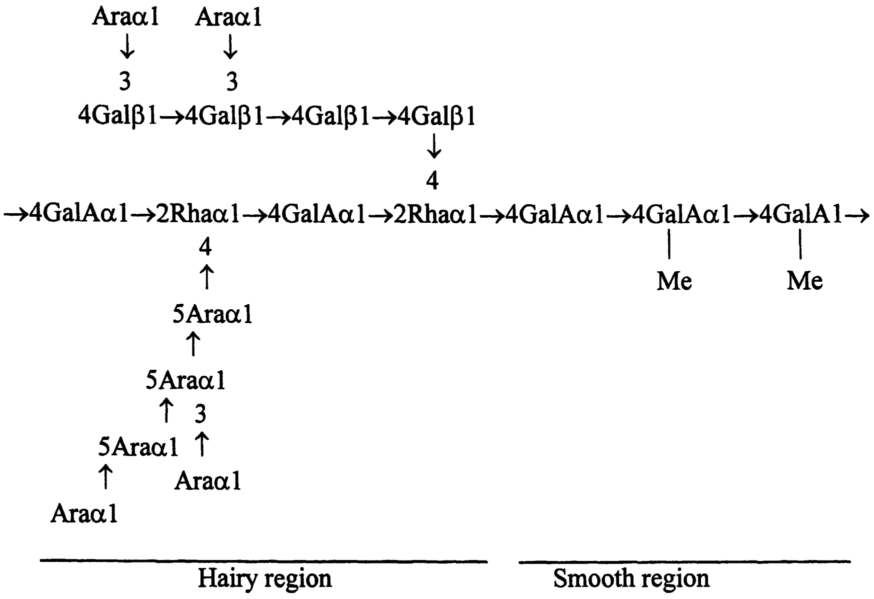
Araα1
Araα1
3
3
4Galβ1→4Galβ1→4Galβ1→4Galβ1
4
→4GalAα1→2Rhaα1→4GalAα1→2Rhaα1→4GalAα1→4GalAα1→4GalA1→
4
Me
Me
5Araα1
5Araα1
3
5Araα1
Araα1
Araα1
Hairy region
Smooth region

Figure 2. Generic structure of pectins. Ara: L-arabinose, Gal: D-galactose GalA: D-galacturonic acid, Me: 6-O-methyl ester, Rha: L-rhamnose.

The most likely source of novel prebiotic oligosaccharides, however, are the pectins. Pectins are complex polysaccharides with some unusual sugar structures. A generic structure for pectin is given in Figure 2.

Pectins are now being investigated as candidate prebiotics. Data presented elsewhere in this volume (*17*) shows that oligosaccharides derived from pectin by controlled enzymatic hydrolysis display an increase in selectivity when used as a fermentation substrate for human fecal bacteria in pH-controlled batch cultures.

A recent study (*22*) has used specific enzymes to produce a range of novel oligosaccharides from plant cell wall polysaccharides (Table III). The fermentation of the oligosaccharides and the parent polysaccharides was then

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investigated in pure culture using a range of intestinal bacterial species from the genera *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium* and *Escherichia coli* and *Klebsiella pneumoniae*. All of the polysaccharides and derived oligosaccharides were fermented by intestinal bacteria to some degree. It is difficult to draw general trends from pure culture data of this kind but the *Bacteroides* species fermented most of the oligosaccharides, particularly the oligorhamnogalacturonans. The *Clostridium* species tested fermented most of the oligosaccharides but had lowest activity against the branched oligoxylans and the oligorhamnogalacturonans. Bifidobacteria fermented all but the oligorhamnogalacturonans and the oligogalacturonans. This latter observation does not accord with the observation of Hotchkiss *et al.* (*17*) that pectic oligosaccharides are bifidogenic using a mixed fecal inoculum, although this study only characterized the bacterial changes at the genus level. This illustrates the limitations of pure cultures in this regard. Similarly it illustrates the need for mixed fecal culture studies to identify bacteria at the species level. The degree of fermentation of the cell wall derived oligosaccharides is, however, encouraging that novel prebiotics might be developed this way.

Table III. Cell Wall-Derived Oligosaccharides (*22*)

Substrate	*Enzyme*	*Derived oligosaccharide*
Soybeans	Endogalactanase	Arabinogalactooligosaccharides
Sugar Beet	Endoarabinanase	Arabino-oligosaccharides
Polygalacturonic acid	Endopolygalacturonase	Galacturonooligosaccharides
Apple	Rhamnogalacturonase	Rhamnogalacturono-oligosaccharides
Wheat Flour	Endoxylanase	Arabinoxylooligosaccharides

Enzymatic Methods of Production

Manufacturing technologies for functional carbohydrates are still developing. New technological approaches are likely to be needed in order to take full advantage of plant cell walls as sources of prebiotics.

Controlled polysaccharide hydrolysis

Polysaccharide hydrolysis is used commercially to manufacture FOS and XOS as prebiotics (*5*). In order to achieve persistent prebiotics, as discussed above, a hydrolysis process, which gives a high degree of control over the molecular weight distribution of the products is needed. Control over the molecular weight distribution will also influence the rheological properties and hence the technological application of the oligosaccharides.

A wide range of degradative enzymes have been described for the hydrolysis of plant cell wall polysaccharides. Rational use of such enzymes with varying substrates can give rise to novel oligosaccharides (*22*). However, technological approaches to the large scale manufacture of oligosaccharides using these enzymes have still to be developed.

A promising approach to the controlled hydrolysis of soluble polysaccharides is the use of endoglycanases in enzyme membrane reactors (EMR). Such reactor systems have been established for the partial hydrolysis of dextran (*23-24*). By controlling factors such as residence time and the ratio of enzyme to substrate, it was possible to convert dextran into different oligodextran preparations with average molecular weights varying from trisaccharide up to 12,000 Daltons. These have proven to be useful prebiotics in gut model systems (*15*) with a high degree of persistence of selective fermentation.

Membrane reactors have also been used for the controlled hydrolysis of pectin (*25*). Low molecular weight oligosaccharide mixtures were made by hydrolysis of high-methyl and low-methyl content pectin and the biological properties of these have been investigated (*17*).

Enzyme membrane reactor systems are not suitable, however, for the hydrolysis of insoluble polysaccharides or for the treatment of crude cell wall materials. For the hydrolysis of insoluble cell wall polysaccharides, immobilized substrate reactors can be used. Such reactors have been developed for the manufacture of XOS from the water-insoluble xylan fraction from corn cobs (*26*). In this reactor configuration, substrate is held in a column and a suitable eluent passed through the bed. Enzyme is pulsed through the column and oligosaccharides and enzyme collected in a fraction collector. Enzyme is then recycled back onto the column. There is also potential for the adaptation of a variety of industrial solid-liquid extraction equipment for use as an enzyme reactor.

In Situ Production During Food Processing Operations

Enzymatic degradation of cell wall polysaccharides occurs during several food processing operations. Xylanases are often used as ingredients in flour improvers for baking (*27*). Partial degradation of arabinoxylans into oligosaccharides has desirable effects on dough rheology, loaf texture and final volume. The liberated oligosaccharides will be prebiotic when they reach the colon, although quantities are likely to be small.

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Pectinases are also receiving increased attention as processing aids in the juice industry (*28*). Complex, potentially prebiotic, oligosaccharides are thus formed in the resultant products. Such enzyme processing could also liberate other functional phytochemicals into the final products, such as antioxidants (*29*).

There is currently no information available on the prebiotic properties, if any of the products of enzymatic processing. An interesting possibility would be the rational development of enzymatic food processing operations specifically designed to increase the levels of prebiotic oligosaccharides in the final products.

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Concluding remarks

The field of prebiotic oligosaccharides is currently a very fertile area for the development of new ingredients and of new products. The current range of prebiotics can conceivably be extended by the rational manufacture of novel molecules from plant cell walls. These new molecules might have enhanced functionality compared with the current generation of prebiotics. The successful development of such ingredients will require research advances in the areas of enzymology, process technology and the use of modern molecular microbiological methods.

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Chapter 5

Pectic Oligosaccharides as Prebiotics

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Pectic oligosaccharides were observed to have bifidogenic prebiotic properties. Pectic oligosaccharides were also found to possess anti-adhesive properties for food pathogen toxins and they stimulated apoptosis of colon cancer cells. Orange peel albedo (white part) was a good source of pectic oligosaccharides with prebiotic properties. Microwave and autoclave extraction produced pectic oligosaccharides with higher degrees of polymerization than those produced with an ultrafiltration dead-end membrane enzyme reactor. We propose that these larger orange albedo pectic oligosaccharides may have greater persistence through the colon, making them excellent candidates for second generation prebiotic product development.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Prebiotic Oligosaccharides

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon (*1*). Typical prebiotics, such as fructo- or galacto-oligosaccharides or lactulose, are selectively fermented, resulting in elevated levels of probiotic bacteria such as bifidobacteria and lactobacilli in the colon. These bacterial genera are considered to be health-promoting for the host (*1*). The commercial prebiotic products currently on the market are produced by enzymatic synthesis or degradation of abundant inexpensive starting materials such as lactose, sucrose, inulin, starch and xylan (*2*). The volume and diversity of the worldwide market for prebiotics is expanding rapidly (*2*) as we become more conscious of the health implications of what we eat. There is great potential for the development of novel second generation prebiotics from agricultural biomass, estimated at over 300 million tons per year in the U.S. alone (*3*), that is rich in rare and unique carbohydrate residues and structures (*4*). Pectin is an acidic polysaccharide abundant in fruit and vegetable processing residues. We estimate that the annual U.S. citrus peel, sugar beet pulp and apple pomace production contains over a million tons of pectin. The vast majority of this biomass is currently sold as cattle feed for less than $0.05 per pound.

Pectic Oligosaccharides

Pectic oligosaccharides are derived from pectin, which is a polysaccharide consisting mainly of a homogalacturonan backbone that is partially methyl-esterified (*4-5*). Homogalacturonan is interrupted periodically by regions of alternating D-galacturonic acid and L-rhamnose residues to which L-arabino-oligosaccharides, D-galactooligosaccharides or arabinogalactan-oligosaccharides are attached (rhamnogalacturonan I). Rhamnogalacturonan II structure is also known in which side chains are attached to a homogalacturonan backbone and these side chains contain unusual sugars, such as apiose, 3-deoxy-D-*manno*-2-octulosonic acid (KDO), 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA) and aceric acid (3-*C'*-carboxy-5-deoxy-L-xylose), in addition to L-rhamnose. Commercial pectin consists of 90% homogalacturonan and 10% rhamnogalacturonan. Food additive quality of commercial pectin is based largely upon molecular weight, degree of esterification (DE) and intrinsic viscosity. However, the branched rhamnogalacturonan content can vary up to 70% in some plant pectins such as sugar beet (*6*). Typically, the highest quality pectins in foods come from citrus fruits such as lime, lemon, grapefruit and orange. The gel-forming properties of sugar beet pectin are

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relatively poor due to its high degree of acetylation, low molecular weight and large proportion of highly branched rhamnogalacturonan I (*7*).

Pectin is considered a soluble dietary fiber with several beneficial gastrointestinal physiological effects including delaying gastrointestinal emptying (*8*), slowing down gut transit time (*9*), reducing glucose absorption (*10*), increasing fecal bulk and lowering cholesterol (*11*). As with other dietary fibers, pectin reaches the large intestine intact where it is fermented by the gut microflora (*12*). During this fermentation process, pectin is degraded to oligosaccharides and smaller metabolites.

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Preparation of Pectic Oligosaccharides

Pectic oligosaccharides (POS) were produced by enzymatic degradation in a continuous ultrafiltration dead-end membrane reactor (*13*). Commercial citrus (high-methoxy, HMP, 60-66% *O*-methyl content) and apple (low-methoxy, LMP, 8% *O*-methyl content) pectins were used to produce POS I (DE = 15%) and POS II (DE = 2%) preparations. An endo-polygalacturonase from *Aspergillus pulverulentus* was used; however, additional enzymatic activities (pectin methyl esterase and pectate lyase) were also present in this commercial enzyme preparation. Based on HPSEC analysis, the apparent average molecular weight of POS I was 3.5 kDa, whereas POS II had a bimodal distribution, with 3.8 kDa and 0.97 kDa average molecular weight values (*13*). Using high performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) with a non-linear potassium oxalate pH 6 mobile phase gradient (*14- 15*), we observed that all of the pectic oligosaccharides in POS I and POS II had a degree of polymerization (DP) of 4 or less compared to a series of oligogalacturonic acids prepared from commercial polygalacturonic acid (PGA)(Figure 1). While the exact carbohydrate composition of POS I and POS II is not yet known, it is clear from the HPAEC-PAD chromatograms that rhamnogalacturonan oligosaccharides as well as oligogalacturonic acids are present due to the shift in retention times of several peaks in the POS chromatograms (Figures 1B and 1C) from those in the PGA hydrolysate chromatogram (Figure 1A). Different levels of methyl-esterification of oligogalacturonic acids in the POS preparations could also explain the shift in retention times.

Prebiotic Properties of Pectic Oligosaccharides

The POS I and POS II preparations were analyzed using controlled pH mixed fecal batch cultures (*16-18*). The POS preparations were generally more bifidogenic than their parent pectin (HMP or LMP) and lower DE pectic oligosaccharides and pectins were more bifidogenic than higher DE material (Table I).

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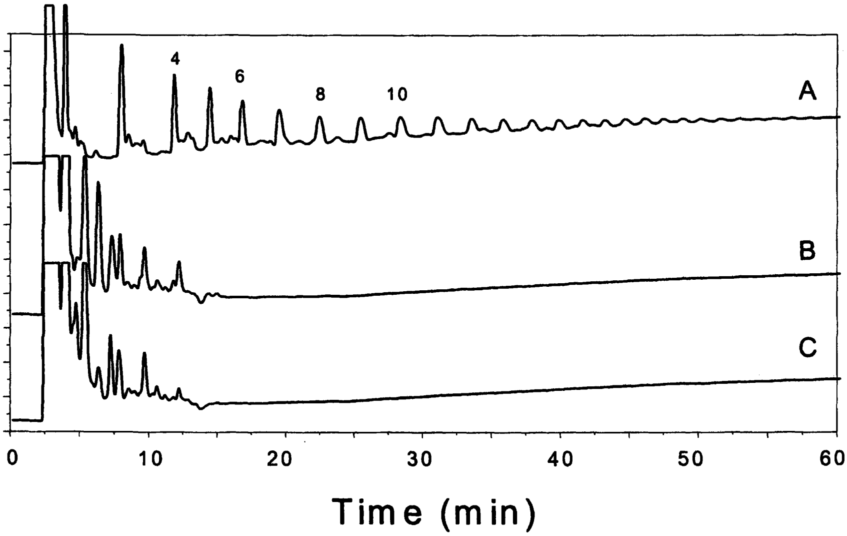

Figure 1. HPAEC-PAD of pectic oligosaccharides. Oligogalacturonic acids (A) were prepared form polygalacturonic acid, while POS I (B) and POS II (C) were prepared by enzymatic degradation of commercial pectins using a continuous ultrafiltration dead-end membrane reactor.

Table I. Prebiotic Properties of Pectins and Pectic Oligosaccharides[a]

	Population (Log10 cells/g)											
	HMP		***POS I***		***LMP***		***POS II***		***OA***		***SB***	
	T_0	T_{24}	T_0	T_{24}	T_0	T_{24}	T_0	T_{24}	T_0	T_{24}	T_0	T_{24}
Total	**10.13**	**10.08**	**10.28**	**10.30**	**10.06**	**10.14**	**10.32**	**10.39**	**9.43**	**9.15**	**9.22**	**9.35**
Bifido	**8.84**	***9.45**	**8.90**	***9.46**	**8.91**	***9.50**	**8.91**	***9.60**	**8.00**	***8.85**	**7.82**	**8.08**
LAB	**8.48**	**8.33**	**8.52**	**8.88**	**8.53**	**8.67**	**8.54**	**8.90**	**7.23**	**7.57**	**7.65**	**7.50**
Bacter	**9.37**	**9.49**	**9.42**	**9.56**	**9.38**	**9.53**	**9.48**	**9.70**	**8.39**	**8.87**	**8.54**	**8.56**
Clostr	**7.61**	**8.06**	**7.52**	**7.57**	**7.61**	**7.86**	**7.72**	**7.59**	**6.42**	**6.56**	**6.40**	**6.42**

[a] Population changes of selected bacterial genera in a pH (6.8) controlled mixed fecal batch culture at 37°C using 1% pectins, 1% pectic oligosaccharides and 1% pectin-rich biomass as carbon sources. Results are the means of six different experiments. No changes in bacterial counts were observed when these carbohydrates were omitted. HMP = high-methoxy pectin, POS = pectic oligosaccharides, LMP = low-methoxy pectin, OA = orange albedo, SB = sugar beet pulp, T_0 = initial time, T_{24} = 24 hours, Total = total bacterial count, Bifido = Bifidobacteria, LAB = lactobacilli, Bacter = Bacteroides, Clostr = Clostridia. *Indicates a significant difference compared to T_0 at $P < 0.01$.

However, the bifidogenic effect of POS was low compared to oligofructose (*18*). Therefore, pectic oligosaccharides had prebiotic properties due to their selective fermentation by human gut bacteria.

Anti-Adhesive Properties

Escherichia coli O157:H7 is a very serious food-borne pathogen that causes a variety of symptoms, ranging from self-limiting watery diarrhea and hemorrhagic colitis to hemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpurea (*19*). These illnesses are caused by the binding of shiga-like toxins, or verocytotoxin (VT), to glycolipid colonic cell surface receptors containing the disaccharide, α-D-Gal*p*-(1→4)-β-D-Gal*p*. Similar oligosaccharides mediate the adhesion of P-fimbriated *E. coli* to uroepithelial cell surfaces (*20*). Digalacturonic acid (α-D-Gal*p*A-(1→4)-D-Gal*p*A) was able to prevent adhesion (50% inhibition at 2.1 mM) of P-fimbriated *E. coli* to uroepithelial cells (*21*). The POS I and POS

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II preparations were able to protect HT29 colon cells from *E. coli* O157:H7 verocytotoxins (*22*). At concentrations at or above 10 mg/ml, significantly higher cell survival of HT29 cells in the presence of VT1 or VT2 was observed with POS I and POS II compared to their parent pectins. We refer to these properties where POS blocked the binding of food pathogen toxins to colonic cells as anti-adhesive.

Anti-Cancer Properties

POS I and POS II preparations also induced apoptosis in HT29 colon cancer cells (*23*). Cell growth was inhibited in the presence of POS and their parent pectins (10 mg/ml). This decrease in cell yield was not due to necrosis nor differentiation, and DNA laddering was observed following agarose gel electrophoresis. These effects were also observed in the presence of butyrate and D-galacturonic acid. Caspase-3 activity, but no alkaline phosphatase activity, was also present.

These potentially anti-cancer effects of pectic oligosaccharides lends further support to other reports of pectin fragments with anti-cancer activity. A pectin fragment was observed to compete with the galectin binding involved in tumor cell metastasis (*24-25*). This pectin fragment, now called GBC-590, is in phase II clinical trials for evaluation in colorectal and pancreatic cancer (GlycoGenesys, Inc.). Another company (EcoNugenics, Inc.) has claimed that modified citrus pectin (a rhamnogalacturonan fragment) enhances the attachment of CD3+ T-cells to tumor cells, thereby, contributing to tumor cell death. Immunostimulatory activity has also been reported for pectin fragments. Citrus pectin fragments inhibited fibroblast growth factor - receptor interaction in the presence of heparin (*26*). A rhamnogalacturonan II fragment also caused up-regulation of Fc-receptors on macrophages (*27*).

Prebiotic Properties of Orange Albedo and Sugar Beet Pulp

Orange albedo (the white part of the peel) was bifidogenic (Table I). However, in the presence of sugar beet pulp, the increase in Bifidobacteria counts was not significant. Orange albedo was the only carbon source evaluated where the total bacterial count decreased. Although this change in the total bacterial count was not significant at 24 hours, the result may indicate that orange albedo limited the growth of pathogenic bacteria while enhancing the growth of health-promoting Bifidobacteria. It is interesting to note that each source of pectin was fermented differently by the gut microflora, leading to distinctly different profiles of bacterial genera.

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Extraction of Pectic Oligosaccharides from Orange Albedo

Orange albedo pectic oligosaccharides were produced by modifying the method published for microwave extraction of pectin (*28*). Shaved Hamlin orange peel was extracted either by the microwave method (*28*) or by autoclave extraction using similar heating, pressure (125°C, 22 psi for 2 min) and cooling conditions. Following precipitation of the pectin with 70% isopropyl alcohol and its collection by filtration, the filtrate was evaporated under reduced pressure to remove the alcohol, neutralized with KOH, passed through a C_{18} solid-phase extraction column (Altex, Harbor City, CA) and lyophilized. The results are shown in Figure 2. The pectic oligosaccharides present in the microwave (Figure

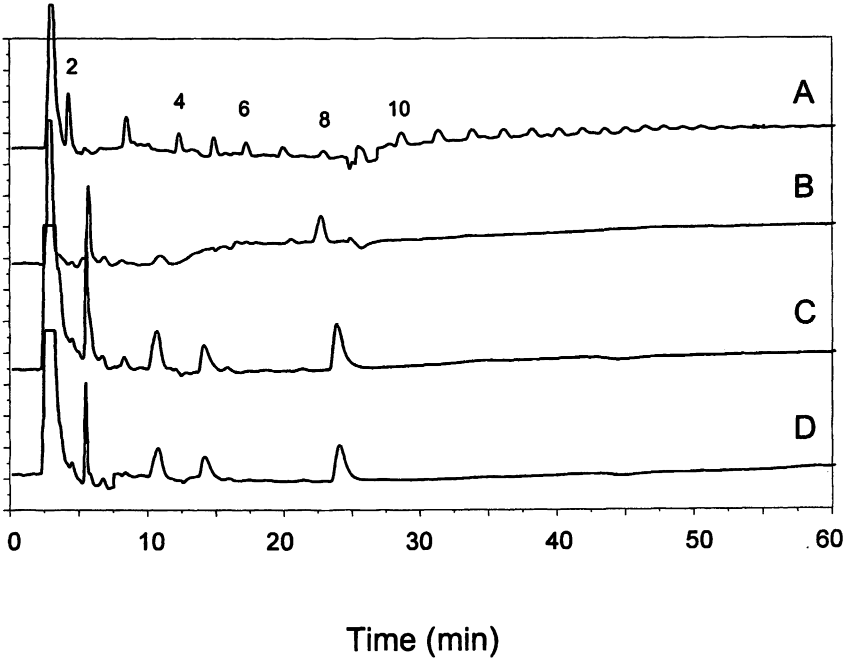

Figure 2. HPAEC-PAD of oligogalacturonic acids (A) and pectic oligosaccharides prepared from orange albedo by microwave (B) and autoclave (C) extraction. The sample in the bottom chromatogram (D) was prepared in sufficient quantity (100 g) for analysis of its prebiotic properties.

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2B) and autoclave (Figure 2C) hydrolysates had a DP up to 8 when directly compared to oligogalacturonic acids in a PGA hydrolysate (Figure 2A). Autoclave extraction of orange albedo produced more pectic oligosaccharide peaks than that produced by microwave extraction. Rhamnogalacturonan oligosaccharides may also be present in these hydrolysates.

Conclusions

Pectic oligosaccharides represent excellent candidates for second generation prebiotics. We have demonstrated that these oligosaccharides are bifidogenic, they block the binding of food pathogen toxins to colonic cells and they stimulate apoptosis of colon cancer cells. Orange peel albedo appears to be an excellent source of prebiotic pectic oligosaccharides. The greater DP of orange albedo pectic oligosaccharides may mean that they persist to more distal regions of the colon where they can exert a greater diversity of health promoting effects.

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Chapter 6

Isomaltooligosaccharides

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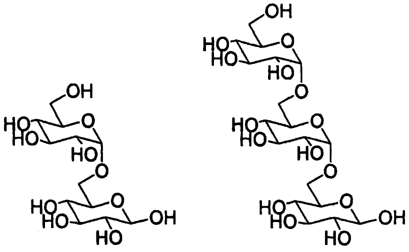
Isomaltooligosaccharides (IMO) have found interest in the fields of food, pharmaceuticals and cosmetics due to their unique properties. Different enzymatic routes are known for their synthesis, some of which are applied industrially. This article present a summary of structural variety, enzymatic synthesis, current technical routes and applications, as well as properties and potential fields for use.

Structures

The typical structure of IMO consists of two or more glucosyl units linked α-1,6 (Figure 1).



Isomaltose IMO

Figure 1. Isomaltooligosaccharide structures.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Other oligosaccharides with isomalto structural units in addition to other structural elements are or may become important with respect to properties and applications. These are IMO with additional glycosidic linkages, such as α-1,2, and /or α-1,3 (Figure 2) (*1-4*). They are formed by certain strains of *Leuconostoc mesenteroides*, notably NRRL B-1299, and different *Streptococcus spp.,* such as *Streptococcus mutans GS5.*

IMO with further functional units, such as alditols, may be of interest since they are non-reducing oligosaccharides, formed via dextransucrase-catalyzed glucosylation of alditols, such as glucitol, mannitol, *O*-α-D-glucopyranosyl-(1→4)-D-glucitol ("maltitol"), or *O*-α-D-glucopyranosyl-(1→6)-D-glucitol and *O*-α-D-glucopyranosyl-(1→6)-D-mannitol (Isomalt^R) (Figure 3) (*6*).

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Analysis

Different HPLC systems may be applied, depending on the constituents of the sample. High sensitivity and separation capacity are offered by the HPAEC-system from the Dionex corporation (column: "CarboPak PA 1", 10 µm, 4 x 250 cm; eluents: A) 0.1 M NaOH and B) 1.0 M NaOAc in 0.1 M NaOH; gradient: in 20 minutes from 100 % A to 97 % A, in 5 minutes from 97 % A to 85 % A, 3 minutes at 85 % A; temperature: 30°C; flow rate: 1.0 mL/min). Other HPLC systems are an aminopropyl-silicagel column for determination of the mono-, di- and trisaccharides (column: "LiChroSpher 100 NH_2" from Merck, Darmstadt, Germany; eluent: acetonitrile/water 80:20 (v/v) or 70:30 (v/v)); or a strong cation exchanger column in the silver form for detection of higher oligosaccharides (column: "Aminex HPX 65 A, BioRad Laboratories, Richmond, USA; eluent: distilled water) (*5*, *6*). Another system used is a C18 column (Bischoff Chromatography) with water as an eluent and a refractometer as detector (*2*).

Other systems have also been applied. A convenient method is TLC (thin layer chromatography), which also can give quantitative results when using a densitometer in the reflective mode (*7*).

Synthesis and kinetics

A direct route to IMO is by dextransucrase with sucrose and glucose as convenient low cost substrates. The molecular weight distribution of the IMO thus formed can be influenced by combining dextransucrase and dextranase (from *Penicillium funiculosum*), increasing the yield of isomaltose to 50% of the sugars produced (*18*). Another route is hydrolysis of dextran, which yields mixtures of IMO. Thus, dextranase has been applied to yield isomaltose and isomaltotriose as the main products with isomaltooligosaccharides up to the

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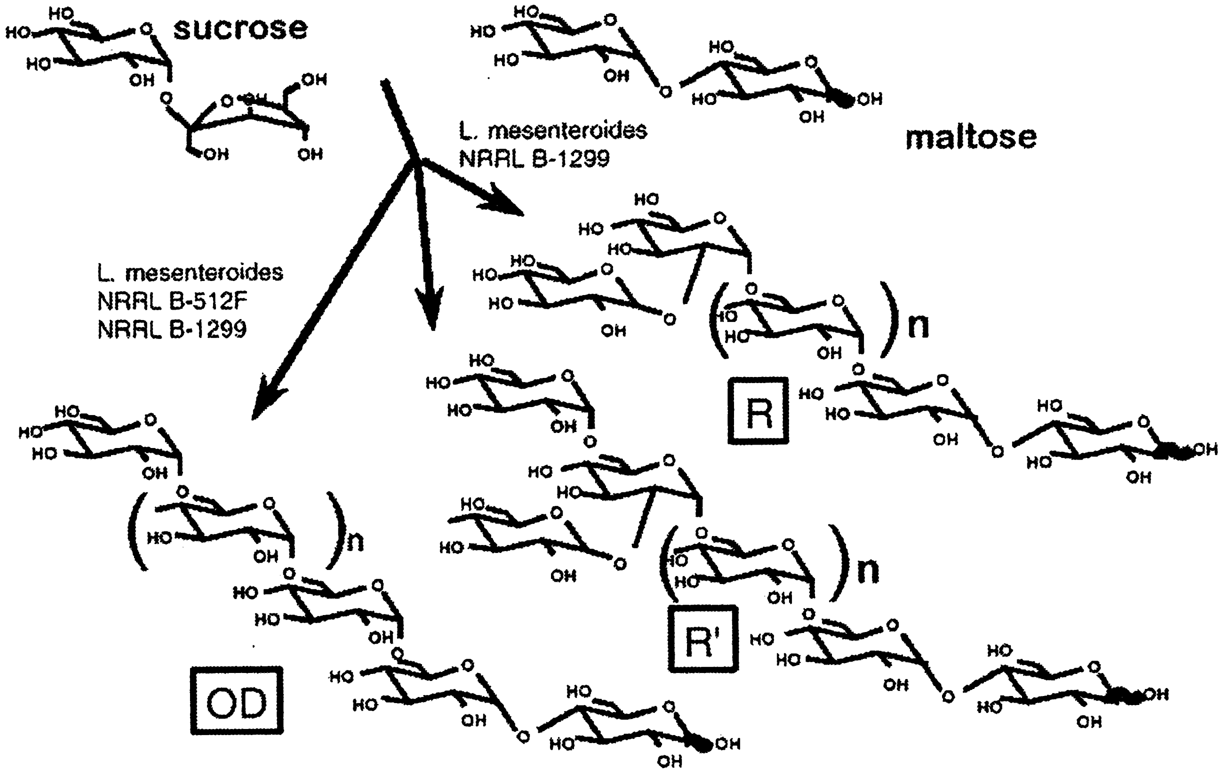
sucrose
maltose
L. mesenteroides
NRRL B-1299
L. mesenteroides
NRRL B-512F
NRRL B-1299
R
R'
OD
n
n
n

Figure 2. Types of oligosaccharides formed by dextran sucrases with maltose as an acceptor.

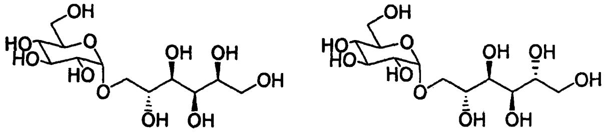

Figure 3. O-α-D-glucopyranosyl-(1→6)-D-glucitol (left) and O-α-D-glucopyranosyl-(1→6)-D-mannitol (right)

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octaose for analytical purposes (*8*). For a chemical synthesis via the Koenigs-Knorr reaction see Igarashi (*9*).

The formation of IMO from sucrose and glucose and other sugars acting as acceptors in glucosyltransfer with dextransucrase has been known for many years (*10-11*) and investigated in more detail by Robyt and coworkers. Kinetic studies were undertaken with the aim to quantify IMO formation and identify conditions for optimal yields (*12,15*). This is essential, since the main reaction catalyzed by dextransucrase is the formation of high molecular weight dextran. Dextran formation can be supressed to a considerable degree by reaction engineering, applying appropriate conditions, primarily with respect to concentrations of sucrose and acceptor, glucose, or maltose or other saccharides, including derivatives. The molecular weight distribution of the products also depends on those conditions.

The glucosylation of D-glucose (Glc) by dextransucrase from *L. mesenteroides* NRRL B-512F with sucrose as the donor substrate yields a homologous series of acceptor products with α-(1→6)-D-glucosidic bonds (isomaltose, isomaltotriose etc., Figure 4) (*15,17*). Yields of acceptor products strongly depend on the concentrations of sucrose as the glucosyl donor and D-glucose as the acceptor (Tables 1 and 2). It further must be taken into account that the acceptor reaction of B-512F dextransucrase with D-glucose is much slower as compared to that with disaccharides such as maltose (by a factor of 2 to 3), isomaltose, (by a factor of 6), which gives the same products, isomaltulose, etc. (*15*). Calculated initial reaction rates as a function of sucrose and D-glucose or maltose concentrations, respectively, are shown in Figure 5. From these data, the appropriate initial concentrations of substrate and acceptor may be estimated, taking into account that the acceptor product mix depends on these concentrations.

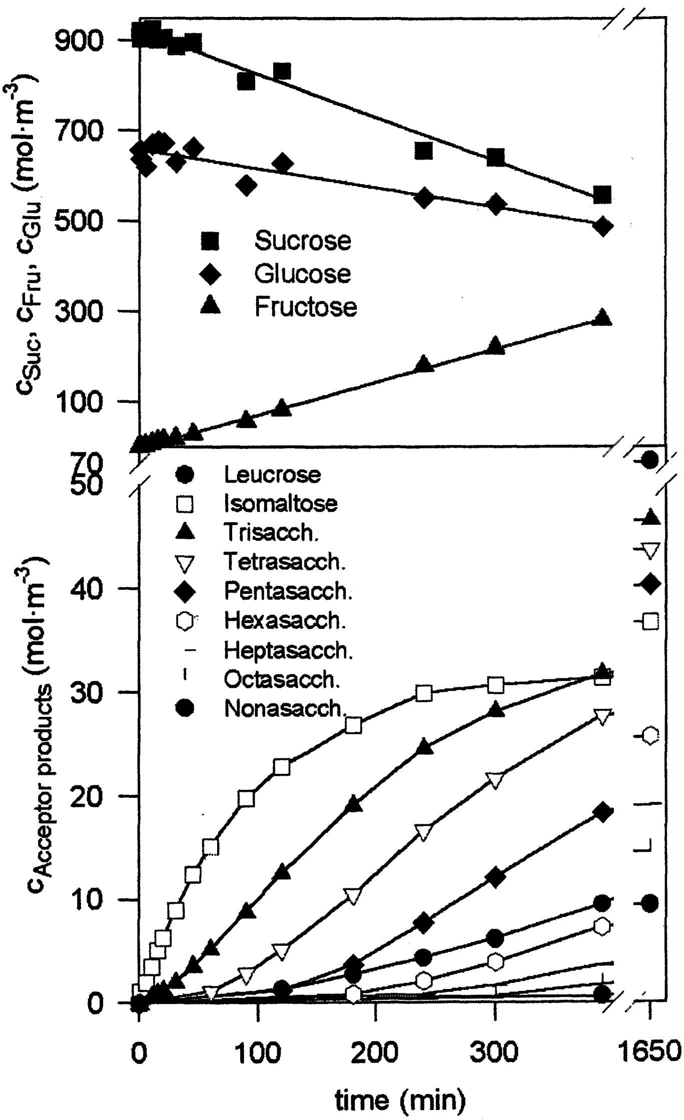

Figure 4. D-Glucose acceptor reaction with dextransucrase (the nonasaccharide gives the lowest acceptor product yield, the symbol being the same as for leucrose).

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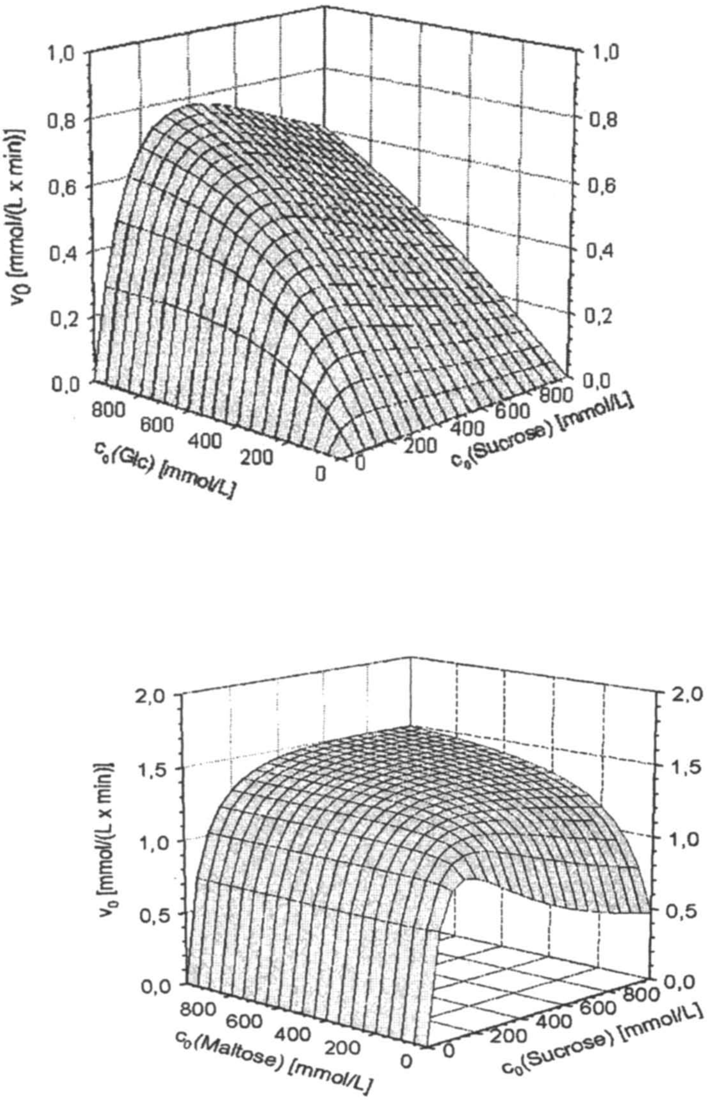

Figure 5. Dextransucrase acceptor reactions, initial overall reaction rates (sum of acceptor products) Top: Glucose; Bottom: Maltose (17)

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Functional IMO can be obtained similarly as described before (*6*). Reaction with non-reducing saccharides (alditols) as acceptors yield IMO with non-reducing end groups. Thus, with glucitol (sorbitol) the products are *O*-α-D-glucopyranosyl-(1→6)-D-glucitol and the homologous series of higher IMO with α-(1→6) bound glucosyl units, up to a DP of 7, with tri- and tetrasaccharides as the main products (Figure 3). One further product is the primary product from glycosylation of the *O*-1 position of glucitol, *O*-α-D-glucopyranosyl-(1→6)-D-gulitol. It must be pointed out, however, that the reactions with alditols from monosaccharides are slower by a factor of about 10 as compared to that with glucose as an acceptor. In contrast, alditols from disaccharides are generally much better acceptors. Thus, with *O*-α-D-glucopyranosyl-(1→6)-D-glucitol and *O*-α-D-glucopyranosyl-(1→6)-D-mannitol, as well as with the commercial mixture (Isomalt^R) (*19*), reaction rates are higher by about a factor of about 10, the main products being tri- and tetrasaccharides, but also yielding IMO with DP up to 7 (the heptasaccharide), depending on the reaction conditions (*6*).

Table 1. Yields (concentration, mM) of Isomaltooligosaccharides

Acceptor product (all data given as approximate conc., mM)	*Low substrate conc. Sucrose 66 mM Glucose 131 mM*	*High conc. Sucrose 394 mM Glucose 920 mM*
Isomaltose	4	35
IM-triose	3	30
IM-tetrose	2	17
IM-pentaose		10

NOTE: it must be considered, that higher IMO consume 2 (triose) to 4 mol (pentaose) of glucosyl units from sucrose

Table 2. Yields (weight %) of Isomaltooligosaccharides

Acceptor product (weight %))	*Glc_0 : 250 mM*	*Glc_0 : 800 mM*
Isomaltose	3	7
IM-triose	4	14
IM-tetrose	7	22
IM-pentaose	11	20

NOTE: IMO-Yields at 526 mM initial sucrose, and two different initial glucose concentrations (Glc_0) (it must be taken into account that weight % differs from mol %)

Standard reaction conditions may be chosen according to experiments reported earlier (*6*): initial sucrose concentration: 526 mM; initial concentration of potential acceptor: 920 mM; dextransucrase activity: 0.80 U/mL; pH 5.4 (maintained by a 25 mM calcium-acetate-buffer); 25°C. 1 unit is defined by the formation of 1 micromole fructose per min, as measured in the "maltose test" with 0.146 M sucrose as the substrate and 0.73 M maltose as an acceptor, other conditions as before (*20*). The reactions were allowed to go for at least 24 hours.

For preparative purposes, concentrations of sucrose and acceptor should be higher, up to the limit of solubility of the reaction mixture. Application of immobilized dextranscrase and a tubular reactor are favourable conditions (*2*, *21*). For separation from fructose as a reaction product and isolation of the IMO chromatographic procedures must be applied (*6*).

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Physiological functions of IMO

Many oligosaccharides have been developed because of beneficial health effects. Isomaltooligosaccharides are useful for prevention and treatment of diseases in immune or other biological functions, microbial infections, and tumors. In this chapter we discuss the wide-range application of isomaltooligosaccharides in the pharmaceutical and food industries.

Gastrointestinal functions

It was shown in recent studies that isomaltooligosaccharids are metabolised *via* intestinal bacteria, like *Bifidobacteria spp.*, which are colonized in the human intestine. The indigestibility is the key function of these oligosaccharides. Digestable carbohydrates such as sucrose, maltose are hydrolysed to monosaccharides by intestinal digestive enzymes and metabolised. In contrast, nondigestible oligosaccharides path through the small intestine tract to the large intestine and enter the fermentation and adsorption process. Intestinal microbes metabolize the oligosaccharides into short-chain fatty acids (SCFAs), for example acetate, propionate, and butyrate, thus lowering the pH in the intestinal tract and preventing the growth of unfavorable organisms, such as *Escherichia coli* and *Clostridium perfringens*.(*22*) The SCFAs are then absorbed from the intestine and eventually degraded to carbon dioxide, thus providing energy. The prebiotic quality of IMO has been found by Rastall *et al.* to increase with the degree of polymerisation up to medium molecular weight (*23*). One of his studies demonstrated the dose-dependent increase of human intestinal *Bifidobacteria spp.* by intake of IMO2 or IMO3. An IMO2 intake of 10 g/day and IMO3 of 5g/day each produced a significant increase of bifidobacterial numbers in feces and the ratio in fecal microflora within 12 days. The metabolic pathway of IMO in healthy men was investigated after ^{13}C-labeled IMO intakes. The $^{13}CO_2$ recoveries were 28.7% in the sedentary test and 60.0% in the exercise test. These recoveries were 70-80% compared with those of maltose. These results indicated that a part of IMO was digested and the residual IMO was fermented by intestinal flora. The energy value of IMO might be about 75% of that of maltose (*24*).

In a clinical study with 7 older men, dietary IMO effectively improved the bowel movement, stool output, and microbial fermentation in the colon without any adverse effects noted (*25*).

Another beneficial function is the increase of absorption and bioavailability of minerals, like magnesium to increase the bone density and relief of anemia (*26*).

Role as cariogenic prophylaxis

Dental caries is mainly caused by the local destruction of teeth by oral bacteria. In their first studies, R. Koch and W. D. Miller thought that bacteria ferment carbohydrates and produce acids to destroy the tissues of the teeth. Miller believed that the oral lactobacilli were the primary cariogenic organisms (*27*). Today it is known *Lactobacillus spp.* do not induce caries. Instead, *mutans streptococci* (MS, *Streptococcus mutans* and related species) have been demonstrated to be the cariogenic bacteria. Glucansucrases from MS transform sucrose to water-insoluble glucan, which sticks to the tooth surfaces and seems to mediate the adhesion of MS. Oligosaccharides like IMOs inhibit glucan synthesis and show a significant prevention of caries (*28*).

IMO's as immunostimulants

It is assumed that prebiotics like IMOs will have immunomodulatory effects on systemic immune response. Studies in mice gave strong evidence that IMO's might stimulate the immune system (*29*). Despite this new found side-effects of IMOs their mechanisms by which oligosaccharides affect the immune functions are still a subject of debate. Rat and human studies demonstrated that SCFA increase the activity of natural killer cells (*30,31*). Butyrate has also been reported as a suppressor of the cytokine induced expression of the transcription factor NFκB in the colonic cell line HT-29 (*32*).

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Production and Application

IMO have been produced in Japan since 1985 by several companies: Showa Sangyo, Nihon Shokuhin Kako, Nikken Kagaku and Hayashibara (*33*,*35*). These are mixtures of oligosaccharides, also called "ALO mixture" (Anomalously Linked Oligosaccharides), with an α-(1→6) glycosidic linkage in addition to α-(1→4) linkages. They are produced from liquefied starch by α- and β-amylase and an *Aspergillus niger* transglucosidase. The syrup contains panose, isomaltose and higher branched oligosaccharides as well as a major amount of D-glucose and a small amount of maltooligosaccharides. It is mildly sweet (40 – 50 % in relation to sucrose), exhibits viscosity-enhancing and water-holding capacity, low cariogenicity and bifidus-promoting properties. The syrup is also used in sake production as a body-giving additive (*33*, *34*).

A mixture of non-digestible isomaltooligosaccharides is formed by dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 which is known to catalyze the synthesis of dextran polymers containing α-(1→2)-linked branched chains. When this specific glucosyltransferase is used in the presence of maltose as an acceptor and of sucrose as glucosyl donor, α-D-glucooligosaccharides are obtained, which contain in adition to α-(1→6) glucosidic bonds α-(1→2) glucosidic bonds at their non-reducing end and a maltose residue at the reducing end (Figure 2) (*3-4*). The presence of these α-(1→2) linkages results in a pronounced resistance of these oligosaccharides to attack by the digestive enzymes of humans and animals (*36*). That is the reason why such α-D-glucooligosaccharides were initially developed as low calorie bulking agents, to be used in food formulations in complement of intense sweeteners (*3*). They are, however, specifically metabolized by the positive intestinal bacterial flora. In contrast to fructooligosaccharides and galactooligosaccharides, these α-D-glucooligosaccharides are not bifidogenic, but they promote the growth of the cellulolytic intestinal flora. In addition, they induce a broader range of glycolytic enzymes than fructooligosaccharides and galactooligosaccharides, without any major side-production of gases and thus any detrimental effect (*37*).

The prebiotic effect of α-D-glucooligosaccharides has been demonstrated for piglets, broilers and calves. The addition, for example, of 0.15% (w/w) of α-D-glucooligosaccharides to young calves' feed results in a 20% decrease of the veterinary costs (*38*).

These oligosaccharides are presently marketed for human nutritional application as food complements, in combination with specific microbial flora and vitamins.

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The prebiotic effect of such α-D-glucooligosaccharides has also been demonstrated at the level of skin microbial flora (*39*), in which lactic bacteria also play a key protective role. This has resulted in the development of dermocosmetic applications for the α-D-glucooligosaccharides, under the trade name BioEcolia.

A European patent (*40*) describes the formation of isomaltooligosaccharides from sucrose using D-glucose as an acceptor. The products contained 10 to 20 anhydroglucose units, the average molar weight being in the range of 2000 to 5000. The molar ratio of sucrose to D-glucose is in the range of 2 to 5. The sucrose is added to the reaction solution of 0.2 to 0.5 M D-glucose and 1000 U of dextransucrase quasi-continuously. The sucrose concentration should not exceed 25% of the total dry substance; the reaction is conducted to a high degree of conversion. The product mixture contains fructose in a molar range corresponding to that of the sucrose added and may be used as a sweetener. Some further data are presented by Pereira *et al.* (*41*) with, however, limited yields of oligosaccharides of up to 45 %. For systematic investigations concerning reaction engineering see Demuth *et al.* (2000).

Conclusions

Considerable research has been devoted to IMO, revealing pathways of formation and reaction mechanisms, as well as kinetics and reaction engineering parameters. Industrial production is established with different product mixtures in Europe and in Japan. Research concerning IMO nevertheless remains an attractive field for further research and development with respect to functional food and pharmaceutical application.

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Chapter 7

Prebiotic Oligosaccharides via Alternansucrase Acceptor Reactions

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Alternansucrase synthesizes an alternating α-(1→3),α-(1→6)-D-glucan via glucosyl transfer from sucrose. It also synthesizes oligosaccharides, containing both types of linkages, when acceptor sugars are present. We have used alternansucrase to synthesize oligosaccharides from maltose, maltodextrins, maltitol, cellobiose, raffinose, melibiose, lactose, gentiobiose and other carbohydrate acceptors. Analysis of the products shows that alternansucrase is better at catalyzing acceptor reactions when compared to dextransucrase, and that the structures of the products differ. Whereas dextransucrase generally makes only a single product from any given acceptor, alternansucrase often makes two or more, and in higher yields. Several of these oligosaccharide acceptor products have been isolated and tested for their ability to support the growth of probiotic bacteria, including selected strains of *Bifidobacterium spp.* and *Lactobacillus spp.* Certain acceptor products supported growth of probiotic strains but did not serve as substrates for undesirable bacteria such as *Salmonella choleraesuis*, *Clostridium perfringens*, or *Escherichia coli.*

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Introduction

There is much current interest in the use of oligosaccharides for their prebiotic activity in foods, animal feeds, and cosmetics. Glucooligosaccharides exhibit certain desirable characteristics in these applications, particularly in their ability to support the growth of probiotic bacteria without the generation of undesirable amounts of gases (*1*). Besides maltooligosaccharides, other glucooligosaccharides include isomaltooligosaccharides, kojioligosaccharides, and mixtures of variously linked saccharides (*2*). One group of glucooligosaccharides that is garnering an increasing amount of interest includes those synthesized from sucrose via glucansucrases (*3*). Glucansucrases are typically extracellular enzymes secreted by bacteria such as *Streptococcus, Lactobacillus*, and *Leuconostoc* spp. They act by transferring D-glucosyl units from sucrose to D-glucose polymers, with the concomitant release of D-fructose. Glucansucrases, including dextransucrase and alternansucrase, have been reviewed in detail (*4-8*).

Of particular interest are the so-called acceptor reactions of glucansucrases. In an acceptor reaction, D-glucosyl units are transferred from sucrose to a hydroxyl-bearing acceptor molecule, resulting in the formation of an α-D-glucopyransosyl acceptor product. Most known acceptors are carbohydrates, although non-carbohydrate acceptors have been described. Alternansucrase, in particular, is known for its ability to catalyze a wide variety of acceptor reactions with various sugars. Unlike dextransucrase (*9-10*), alternansucrase often forms two or more linkage types with a single acceptor (*3, 11-12*). It has also been noted that alternansucrase carries out acceptor reactions with a wider variety of acceptors with greater yields (*13*).

There are some important facts about acceptor reactions that must be noted. Firstly, each acceptor differs in its ability to divert the transfer of D-glucosyl units away from glucan synthesis and into the formation of an acceptor product. This differential reactivity is often referred to as acceptor strength. A good acceptor may be able to divert nearly all glucosyl transfer away from glucan formation and into the synthesis of oligosaccharides. Secondly, many acceptor products are themselves capable of acting as acceptors. For example, it often happens that a monosaccharide acceptor yields a disaccharide product which in turn yields a trisaccharide product, etc. Thirdly, the extent of an acceptor reaction relative to polymerization depends on the concentrations of sucrose and acceptor, and especially on their relative ratio. At a relatively high sucrose:acceptor ratio, more glucosyl units are transferred into glucan and higher DP (degree of polymerization) acceptor products. At a low sucrose:acceptor ratio, the predominant reaction product is that resulting from the transfer of a single glucosyl unit to the acceptor.

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This phenomenon is very useful in optimizing yields of variously sized oligosaccharides (*3, 12*).

This paper describes our survey of a number of acceptors with alternansucrase, the isolation and structural identification of several of the products, and the prebiotic activity of some selected acceptor products.

Materials and Methods

Alternansucrase

The alternansucrase preparation used in these experiments was isolated from *Leuconostoc mesenteroides* NRRL B-21297, a proprietary strain that secretes alternansucrase at enhanced levels, and does not produce dextransucrase (*14-15*). Bacteria were grown at 28°C in a stirred, 10-liter batch fermentor in the medium previously described (*16*). After inoculation of 9.5 L medium with 0.5 L of 24-hour culture, the contents were agitated at 150 rpm, with aeration at 0.4 L/min. The pH was maintained at 5.5 by addition of 1M NaOH. Bacterial cells were removed after 24 hours by centrifugation at 16,000 x g for 20 minutes. The culture fluid was concentrated by tangential flow ultrafiltration over 100,000 MW cutoff membranes, and subsequently diafiltered over the same membranes against 20 mM pH 5.4 sodium acetate buffer containing 0.01% (w/v) sodium azide. The 10-fold concentrated and dialyzed culture fluid was used as alternansucrase without further purification, and contained 1.5 units/mL when measured radiometrically for alternan-synthetase activity (*11*).

Carbohydrates

Carbohydrates were purchased from Sigma Co. (St. Louis, MO, USA) except for the following: L-fucose, L-rhamnose, raffinose, 3-*O*-β-D-galactopyranosyl-D-arabinose, 2-deoxy-D-galactose, D-arabinose, L-glucose, D-psicose, *N*-acetyl-D-mannosamine, α-methyl-D-galactopyranoside, and β-methyl-D-galactopyranoside (Pfanstiehl Laboratories, Waukegan, IL, USA), planteose, gentianose, and stachyose (Fluka AG), L-arabinose and melibiose (Fisher Scientific), β-octyl-D-glucopyranoside (Pierce Chemicals, Rockford, IL), β-dodecyl maltoside (Calbiochem Co., San Diego), melezitose (General Biochemicals, Chagrin Falls, OH, USA), turanose (Nutritional Biochemical Co., Cleveland, OH, USA), lactulose (Applied Science Labs., State College, PA, USA), D-tagatose (P-L Biochemicals, Milwaukee, WI, USA), lactose (Difco Co., Detroit, MI, USA), maltitol (Towa Chem. Ind., Tokyo), xylsucrose (a gift from Ensuiko Sugar Co., Yokohama, Japan), theanderose (purchased from Wako Pure Chemicals and purified as previously

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described (*18*)), and kojibiose, kojitriose, 6-*O*-α-D-glucopyranosyl-α,α-trehalose and 6,6'-di-*O*-α-D-glucopyranosyl-α,α-trehalose (gifts from Hayashibara Biochemical Co., Okayama, Japan). Leucrose, cycloalternan, and the alternan trimer 3-*O*-α-D-glucopyranosyl-6-*O*-α-D-glucopyranosyl-D-glucose were prepared as previously described (*18*). Neosugar™, a fructooligosaccharide mixture, was a gift from Meiji, Ltd., Saitama, Japan.

Analytical Methods

Carbohydrate mixtures were analyzed by thin-layer chromatography (TLC) as previously described (*11*). Preparative HPLC was performed at room temperature using a C-18 column with water as the eluent. Detection was by either refractive index or optical rotation. Oligosaccharides were separated by size using a Bio-Gel P-2 (fine mesh) column (5×150 cm), eluted with water under gravity flow. Detection was by TLC. Nonreducing products such as sugar alcohols and methyl glycosides were separated by ion-exchange chromatography over Dowex 1X4-200 mesh anion exchange resin in the hydroxyl form, using water as the eluent. Structural analyses of oligosaccharides were carried out by methylation and NMR as previously described (*19*). Total carbohydrate content was measured by the phenol-H_2SO_4 method (*20*), and reducing sugar concentrations were determined by an automated alkaline ferricyanide technique (*21*).

Acceptor Reaction Conditions

Acceptor reactions were carried out at room temperature in 20 mM pH 5.4 sodium acetate buffer containing 0.01% sodium azide. For quantitative comparisons, reaction mixtures contained 45 μL of 10% (w/v) sucrose, 45 μL of 10% (w/v) acceptor sugar, and 10 μL of alternansucrase solution. Reactions were monitored by TLC, and were judged to be complete when all of the sucrose had been consumed. Upon completion of the reaction, each was mixed with 0.1 mL of ethanol, and the alternan thus precipitated was pelleted by centrifugation. The alternan pellets were redissolved in 0.3 mL water, reprecipitated with 0.5 mL ethanol, and redissolved in 1 mL water. These aqueous solutions were analyzed for total carbohydrate content. Several samples were also analyzed by densitometry of the thin-layer plates, and it was found that those results correlated well with measurements of alternan formation as an indication of relative acceptor strengths. This is in agreement with the findings of Robyt and Eklund (*9*) that oligosaccharide formation is inversely proportional to glucan formation once all sucrose has been consumed.

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Bacterial Cultures

All bacterial cultures were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and included *Bacteroides thetaiotaomicron* ATCC 29148, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium breve* ATCC 15698, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium longum* ATCC 15707, *Bifidobacterium pseudocatenulatum* ATCC 27919, *Clostridium perfringens* ATCC 13124, *Enterobacter aerogenes* ATCC 35028, *Escherichia coli* ATCC 8739, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393, *Lactobacillus rhamnosus GG* ATCC 53103, and *Salmonella choleraesuis (typhimurium)* ATCC 14028.

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Culture Media and Growth Conditions

B. thetaiotamicron was grown on a defined medium (*22*), *Bifidobacterium* species were grown on DSM 58 medium (*23*), *C. perfringens* was grown on TGY medium (*24*), *E. aerogenes*, *E. coli*, and *S. choleraesuis* were grown on a modified defined medium (*25*), and *Lactobacillus* species were grown on MRS (*26*) medium.

For carbohydrate utilization tests, various oligosaccharide preparations were added to each medium as the sole carbohydrate source at a concentration of 0.5%. Media were then filter-sterilized using a 0.2µm membrane (Fisherbrand, Fisher Scientific) and added to sterilized 16 mm screw-cap test tubes to a volume of 9.75 ml. Oxygen was removed by adding Oxyrase to each test medium according to manufacturers instructions (Oxyrase, Inc., Mansfield, OH). Inocula for the test media were prepared by cultivating each microorganism for 24-48 hrs in the appropriate medium containing 0.5% glucose as the sole carbohydrate source. Each test medium was then inoculated with 0.25 ml of the 24-48 hr cultures. Test media containing no carbohydrate were also inoculated with each microorganism as a control to account for glucose carry-over. Following inoculation of the test media, cultures were incubated anaerobically for up to 4 days at 37°C. Anaerobic conditions were established using the Gas Pak system (Becton Dickinson Microbiology Systems, Sparks, MD).

Carbohydrate Utilization

Each bacterial species was tested for its ability to utilize the maltose, melibiose, and raffinose acceptor products. Carbohydrate utilization was determined by measuring growth (Abs. at 600nm, DU-64 Spectrophotometer, Beckman, Schaumburg, IL) and acid production (pH meter, Corning, New York, NY) from each organism following the incubation period. Each carbohydrate utilization test was performed in triplicate.

Results and Discussion

Comparison of Acceptors

The results of quantitative comparisons of relative acceptor strengths are shown in Table I. Some general observations may be made. The best acceptors tended to be α-linked di- or trisaccharides of D-glucose, or in the case of maltitol, the reduced analogue thereof. Except for gentiobiose, the β-linked glucodisaccharides were less reactive. This suggests some degree of steric hindrance may exist in the β-linked disaccharides, which is alleviated by the extra degree of rotational freedom of the β-(1→6) linkage. Why gentiobiose is a better acceptor than isomaltose is a mystery. Sugar alcohols were very poor acceptors, with barely detectable levels of acceptor products observed on TLC. α,α-Trehalose was not an acceptor, despite the fact that it contains two α-linked D-glucopyranosyl units. Its derivatives, 6-*O*-α-D-glucopyranosyl-α,α-trehalose and 6,6'-di-*O*-α-D-glucopyranosyl-α,α-trehalose, were both relatively good acceptors.

The products of alternansucrase acceptor reactions with several glucodisaccharides have been described previously (*3, 11-13*). It has been noted that α-(1→6) linkages were usually formed first, but if an α-(1→6) linkage was already present, then either an α-(1→6) or an α-(1→3) linkage was formed (*11*). The β-linked disaccharide cellobiose presents a somewhat different picture. In that instance, two different products were initially formed. One was the result of the formation of an α-(1→6) linkage to the D-glucosyl unit at the nonreducing end of the disaccharide, and the other arose from the formation of an α-(1→2) linkage to the reducing-end glucosyl unit (*13*). Only the latter compound has been isolated from dextransucrase acceptor reactions with cellobiose (*10, 27*). Higher DP oligosaccharides were also formed by subsequent glucosylations.

Maltitol Product

We found maltitol, like maltose, to be an excellent acceptor for alternansucrase. Not surprisingly, the initial product is panitol, analogous to the formation of panose

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Table I. Comparison of Acceptor Strengths with Alternansucrase, as Measured by Relative Amount of Alternan Formed.

Monomers	*Alternan (Rel. %)*	*Di- & Oligomers*	*Alternan (Rel. %)*
dulcitol	101	α,α-trehalose	102
mannitol	100	lactulose	91
myo-inositol	99	galactosyl-arabinose	90
D-arabinose	99	melezitose	90
D-lyxose	99	*cyclo*Glc$_4$ (cycloalternan)	90
xylitol	97	stachyose	89
D-sorbose	97	kojitriose	86
L-rhamnose	96	β-dodecyl maltoside	83
β-methyl-D-xyloside	96	leucrose	81
β-methyl-D-galactoside	94	sophorose	80
L-fucose	94	lactose	79
D-ribose	93	planteose	76
N-acetyl-D-mannosamine	93	cellobiose	72
L-lyxose	92	palatinose	69
D-altrose	92	raffinose	67
D-mannose	90	gentianose	66
N-acetyl-D-glucosamine	90	maltotriose	65
2-deoxy-D-galactose	89	laminaribiose	58
β-methyl-D-mannoside	89	turanose	57
N-acetyl-D-galactosamine	88	6-*O*-α-D-Glc-trehalose	56
L-sorbose	88	6,6'-di-*O*-α-D-Glc-trehalose	54
D-xylose	86	isomaltotriose	50
D-psicose	85	melibiose	45
D-galactose	83	isomaltose	44
sorbitol	82	theanderose	42
2-deoxy-D-ribose	81	6''-*O*-α-D-Glc panose	36
D-fructose	78	3'-*O*-α-D-Glc isomaltose	31
D-talose	77	kojibiose	27
L-arabinose	75	panose	25
D-allose	75	gentiobiose	25
α-methyl-D-galactoside	73	nigerose	23
α-methyl-D-mannoside	70	maltitol	18
D-tagatose	65	maltose	11
L-glucose	63		
D-quinovose	61		
β-methyl-D-glucoside	60		
2-deoxy-D-glucose	59		
D-glucose	53		
β-octyl D-glucoside	52		
α-methyl-D-glucoside	20		

from maltose (*11*). Higher DP products were also formed, but have not yet been isolated.

Gentiobiose Acceptor Product

Gentiobiose was a surprisingly good acceptor for alternansucrase. When tested under identical conditions, it was even better than isomaltose (Table I). Only a single initial product was formed, which proved to be α-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-D-glucose. Higher DP products were formed by subsequent glucosylations, but have not yet been identified. When the acceptor reaction conditions were such that the initial sucrose concentration was 6% (w/v) and the initial gentiobiose concentration was 3%, the total yield of trisaccharide was 72% based on gentiobiose, and the total oligosaccharide yield up to DP 5 was 88% based on gentiobiose. Only maltose, maltitol, nigerose, and α-methyl-D-glucopyranoside were better acceptors for alternansucrase. Gentiobiose is of interest as a food additive and prebiotic, but has a bitter taste. Glucosylation by alternansucrase may result in a less bitter product that would be more palatable in certain food applications.

Products from α-D-Galactopyranosides

Both melibiose and raffinose were relatively good acceptors. Compared with *L. mesenteroides* NRRL B-512F dextransucrase under identical conditions, the acceptor product yields with alternansucrase were much higher as determined by the size and intensity of product spots on TLC analysis (data not shown). Acceptor products from melibiose, raffinose and α-methyl-D-galactopyranoside were isolated and characterized by methylation analysis and NMR.

Alternansucrase gave two products by acceptor reaction with α-methyl-D-galactopyranoside. These were separated by ion-exchange chromatography. Methylation and NMR analysis showed these products to be α-D-glucopyranosyl-(1→4)-α-methyl-D-galactopyranoside (*28*) and α-D-glucopyranosyl-(1→3)-α-methyl-D-galactopyranoside (*29*) in a molar ratio of approximately 2.5:1. By comparison, the only product reported to be synthesized by B-512F dextransucrase is α-D-glucopyranosyl-(1→4)-α-methyl-D-galactopyranoside (*30*).

When the alternansucrase acceptor reaction with melibiose was analyzed by TLC, only a single initial product was observed on TLC. This product was isolated by gel filtration chromatography and analyzed by NMR and methylation. It was found to be α-D-glucopyranosyl-(1→3)-α-D-galactopyranosyl-(1→6)-D-glucose, although trace contaminants in the GC-MS of the methylation products and minor NMR peaks suggested the presence of α-D-glucopyranosyl-(1→4)-α-D-

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galactopyranosyl-(1→6)-D-glucose as a minor product (<10%). Melibiose has been reported to be an acceptor for B-512F dextransucrase (*31*), but the structure of the product has, to the best of our knowledge, never been reported. We isolated the melibiose acceptor product from B-512F dextransucrase and analyzed it by NMR and methylation. The results indicate that dextransucrase synthesizes α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→6)-D-glucose from melibiose, which is analogous to the acceptor product arising from α-methyl-D-galactopyranoside (*30*).

Raffinose proved to be an interesting acceptor for alternansucrase. Raffinose itself is of somewhat limited solubility in water. We discovered that it is possible to react sucrose solutions with a saturated slurry of raffinose in the presence of alternansucrase. As the enzyme glucosylates raffinose, the product is solubilized to a greater extent than the raffinose, so that eventually nearly all of the raffinose becomes solubilized via glucosylation. Two initial acceptor products were formed and were separated by preparative HPLC. Methylation and NMR showed these to be α-D-glucopyranosyl-(1→4)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside and α-D-glucopyranosyl-(1→3)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside in a 9:1 molar ratio. Thus, we find that in each case, α-D-galactopyranosides are glucosylated at both positions 3 and 4 by alternansucrase, with the relative ratio being variable. Interestingly, the dextransucrase acceptor product arising from raffinose is reported to be glucosylated at position 2 of the α-D-glucopyranosyl residue (*32*).

Fructose-containing Products

Whenever sucrose serves as the substrate for glucansucrases, fructose is released. Fructose acts as an acceptor, giving rise mainly to leucrose (*11, 33*). It has been stated that leucrose is a poor acceptor for dextransucrase (*34*) and alternansucrase (*11*). This was based on the absence of higher DP products in reactions of the enzyme with sucrose alone. In such cases, leucrose formation is relatively low, usually less than 10% (*9, 11*). At the early stages of reaction, fructose concentrations are relatively low, so leucrose concentrations would not build up to significant levels until later in the reaction. However, when leucrose was added as an acceptor at the same initial level as other disaccharides we tested, we observed a number of acceptor products on TLC. This is reflected in Table I, where leucrose resulted in a 19% reduction in the amount of alternan formed, presumably correlating to the diversion of 19% of glucosyl residues into oligosaccharide synthesis. Palatinose (isomaltulose), which is another acceptor product formed from fructose (*35*), also serves as an acceptor for alternansucrase (Table I). In fact, it is even better than leucrose, yielding the same amount of

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product as raffinose or cellobiose. The products arising from leucrose and palatinose have not been structurally characterized.

Other Products

Other interesting acceptors include α-methyl-D-mannopyranoside and lactose. α-Methyl-D-mannopyranoside gave rise mainly to α-D-glucopyranosyl-(1→6)-α-methyl-D-mannopyranoside. Other, minor products, were also formed, and are currently under study. Lactose, however, presents a much more complicated picture. It was found to yield a mixture of products which have proven difficult to separate completely. Methylation analysis suggests the presence of α-D-glucopyranosyl-(1→2) linkages to both the D-glucopyranosyl and D-galactopyranosyl moieties, but results are at this time too ambiguous to interpret with any degree of certainty.

Rates of Acceptor Reactions

Glucansucrases may be viewed as possessing two distinct activities, based on the two-step reaction they catalyze. They may be viewed as glucan-synthesizing enzymes, in which case the activity is measured by the rate of synthesis of polymeric D-glucan. They may also be viewed as sucrases, in which case the activity would be most accurately measured by the rate of disappearance of sucrose. Although measuring sucrose consumption is tedious, measuring the corresponding release of reducing sugar (mainly fructose) is simple. Under ideal conditions, the two methods should yield the same results. A problem arises, however, when acceptor sugars are added to the reaction mixture. Ignoring for the moment that most acceptors also happen to be reducing sugars, the main problem lies in the fact that acceptor reactions will divert glucosyl transfer away from glucan synthesis and into the formation of low-molecular weight oligosaccharides. A good acceptor will act as an inhibitor of glucan synthesis, and any assay that measures glucan synthesis will characterize acceptors as inhibitors (e.g., *36*). However, when the rate of sucrose consumption or reducing sugar release is measured, the inhibition of glucan synthesis is not a factor.

We observed in many of our acceptor reactions that sucrose disappeared much more rapidly in the presence of good acceptors than when no acceptor was present. To demonstrate this phenomenon, we assayed alternansucrase in the presence and absence of two different nonreducing acceptors, measuring the rate of release of reducing sugar (i.e., fructose) as an indicator of sucrase activity. Since no detectable D-glucose is released in the reaction, it can be assumed that this reflects

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an accurate measurement of the total rate of glucosyl transfer activity, including both alternan and oligosaccharide synthesis.

Figure 1 shows the rates of alternansucrase action in the absence and presence of two good acceptors, α-methyl-D-glucopyranoside and maltitol. In each case, the rate is drastically enhanced by the presence of acceptor. This does not necessarily conflict with the results of Tanriseven and Robyt (*36*), as different enzymes were used and the rates of different reactions were measured. In fact, their finding of a separate acceptor binding site suggests the possibility of allosteric interactions with acceptors. It may be concluded that some good acceptors accelerate the rate of glucosyl transfer by alternansucrase. This is a welcome advantage for the synthesis of acceptor products.

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Prebiotic Activity of Oligosaccharide Mixtures

Some of the acceptor product mixtures were tested for their ability to support the growth of probiotic bacteria, as well as undesirable bacteria. Mixtures were tested, rather than pure products, since it is likely that the cheaper mixtures would be used in any commercial applications. The maltose acceptor product mixture contained products mainly in the DP 3-7 range. Melibiose products in the DP 2-4 range (approx. 90% DP 3) , and raffinose products in the DP 3-5 range (approx. 90% DP 4) were also tested.

The results, shown in Table II, compare favorably with those of Neosugar™, a commercial fructooligosaccharide mixture. The most notable result is that the alternansucrase acceptor products were more selective for *Bifidobacterium spp.*, and did not support much growth of *Lactobacillus spp.* This may or may not be an advantage, depending on the intended application. The alternansucrase products from melibiose and raffinose were also better in the sense that they did not support growth of the gas-producing anaerobe *Clostridium perfringens.*

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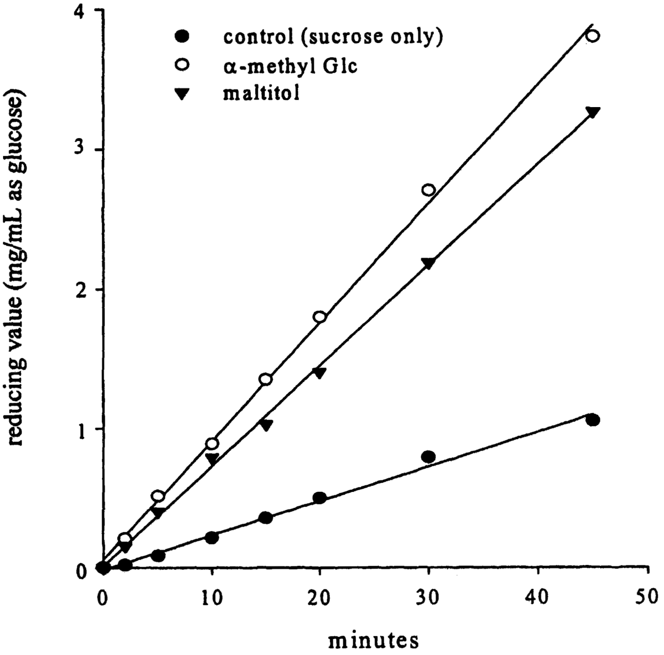

Figure 1. Activation of alternansucrase by acceptors. Rates of release of reducing sugar from sucrose in the presence and absence of nonreducing acceptors. Conditions: 0.9 mL 10% (w/v) sucrose, 0.9 mL 10% (w/v) acceptor or buffer, and 0.2 mL alternansucrase, 1.5 U/mL, all in 20 mM pH 5.4 sodium acetate buffer at 25°C. Reactions were terminated by dilution in 10 volumes of 0.5 M sodium carbonate, and assayed for reducing sugar as described in materials and methods.

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Table II. Growth of selected bacteria on oligosaccharides.

Genus and species	*Maltose acceptor products*	*Melibiose acceptor products*	*Raffinose acceptor products*	*Neosugar (FOS)*
Bacteroides thetaiotamicron	+	++	+	++
Bifidobacterium adolescentis	+++	+++	+++	+++
Bifidobacterium breve	+	-	-	+++
Bifidobacterium infantis	-	+	+	+++
Bifidobacterium longum	-	+	+	+++
Bifidobacterium pseudocatenulatum	+++	+++	+++	+++
Lactobacillus acidophilus	-	-	-	+++
Lactobacillus casei	+	-	-	-
Lactobacillus rhamnosus GG	-	-	-	-
Enterobacter aerogenes	-	+	-	++
Escherichia coli	-	+	-	++
Clostridium perfringens	+	-	-	-
Salmonella cholerasuis (Typhimurium)	-	-	-	-

+++	OD > 0.60, high growth
++	OD 0.31 - 0.59, medium growth
+	OD 0.10 - 0.30, low growth
-	OD < 0.10, no growth

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Chapter 8

Glucansucrases: Structural Basis, Mechanistic Aspects, and New Perspectives for Engineering

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Glucansucrases are remarkable transglycosidases, which catalyse the synthesis of D-glucans containing various types of glucosidic linkages from sucrose substrate. Among them, four enzymes retained our attention because of their distinct specificities : i) amylosucrase from *Neisseria polysaccharea,* ii) alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355, iii) dextransucrase from *L. mesenteroides* NRRL B-1299, iv) dextransucrase from *L. mesenteroides* NRRL B-512. From sequence analyses, these glucansucrases can be classified in the clan GH-H of glycoside hydrolases, which includes the families 13, 70 and 77. The diversity of glucansucrase specificities and their ability to modify a large number of acceptor molecules by glucosylation offer multiple possibilities for the synthesis of novel polymers or oligosaccharides. To improve the understanding of their action mode and extend their applications, relationships between glucansucrase structure and function are investigated. This presentation describes our recent findings on the selected enzymes and the new perspectives offered for designing glucansucrases with improved properties.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
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Introduction

Glucansucrases are found in bacteria belonging to various genera: *Leuconostoc, Lactococcus, Streptococcus, Neisseria* (*1-3*). These enzymes are transglycosidases and their primary function consists in synthesizing high molecular weight glucans from sucrose. A large variation exists in the type of glycosidic linkages that can be formed.

Glucansucrases are very attractive for the synthesis of novel oligosaccharides and polymers (*4*). They use sucrose, a widely available and low cost substrate. They liberate D-fructose, a valuable by-product, and have the ability to synthesize glucan or oligosaccharides in high yields. The first step of the reaction consists of the formation of a glucosyl-enzyme intermediate with a concomitant release of fructose. Once the glucosyl-enzyme is formed, the glucosyl moiety can be transferred to the polymer chain (that is the main reaction), to water (hydrolysis reaction) or to fructose, leading to the formation of sucrose isomers. In addition, when an acceptor is added to the reaction mixture, the enzymatic activity can be redirected from glucan synthesis toward acceptor glucosylation (*5*). By these means and using glucansucrases of distinctive specificities, it is possible to synthesize various types of oligosaccharides or glucoconjugates. However, the use of this group of enzymes for large-scale manufacture of novel products is still limited by several factors such as enzyme selectivity, stability and, in some cases, efficiency.

To overcome these limitations and further enlarge the applications of glucansucrases, the aim of our research work is to generate, via protein engineering, improved glucansucrases for novel glucan, oligosaccharide or gluconjugate production. For this purpose, two approaches are considered : rational design, which involves site-directed mutagenesis guided by three dimensional structure analysis and *in vitro* molecular evolution which requires the development of efficient screening methods for the isolation of improved catalysts. In the present paper, recent updates of this work will be presented.

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Glucansucrase Selection

One of the targets of our work being the understanding of the glucansucrase mode of action and the identification of the structural determinants that define the specificity of each enzyme, structure function relationship studies have been centered on four glucansucrases selected on the basis of their distinctive specificities: dextransucrase from *L. mesenteroides* NRRL B-512F, alternansucrase from *L. mesenteroides* NRRL B-1355, dextransucrases from *L. mesenteroides* NRRL B-1299 and amylosucrase from *Neisseria polysaccharea* (Table 1).

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Table I. Specificity of Selected Glucansucrases

Name	*Producing strain*	*Variety of glycosidic bonds in the glucan synthesized*				
		% α-1,6	*% α-1,3*	*% α-1,2*	*% α-1,4*	*ref*
Dextransucrase DSR-S	*L. mesenteroides* NRRL B-512F	95	5			1
Dextransucrase DSR-E	*L. mesenteroides* NRRL B-1299	66	1	27		1
Alternansucrase ASR	*L. mesenteroides* NRRL B-1355	54	46			1
Amylosucrase AS	*Neisseria polysaccharea*				100	6

Dextransucrase from *L. mesenteroides* NRRL B-512F synthesizes an almost linear water-soluble dextran with 95% α-1,6 linkages in the main chains and 5% α-1,3 branch linkages. Alternansucrase produces another very atypical glucan named alternan composed of glucosyl residues linked by alternating α-1,6 and α-1,3 linkages. The dextran produced by *L. mesenteroides* NRRL B-1299 glucansucrase contains a high percentage of α-1,2 branch points. Finally, amylosucrase synthesizes an insoluble amylose-like glucan polymer exclusively formed of α-1,4 linkages.

Advantages of Glucansucrases For Novel Sugar Synthesis

One of the key properties of glucansucrases is that glucansucrases conserve their specificity when they catalyze oligosaccharide synthesis (*7-9*). Usually, similar glycosidic bonds are found in the glucan polymer and in the acceptor reaction products. This property was applied to the production of dietary and prebiotic oligosaccharide containing α-1,2 linkages using dextransucrase from *L. mesenteroides* NRRL B-1299. A useful method was provided for producing these oligosaccharides on an industrial scale in a plug flow reactor with immobilized catalyst (*10-11*). With amylosucrase, the specificity is also conserved during transfer onto an acceptor. This enzyme is a very useful tool for glucose, maltooligosaccharides or glycogen modification. In the presence of glucose or maltooligosaccharide acceptors, amylosucrase catalyses the synthesis of maltooligosaccharides of increasing degree of polymerization (DP). Amylosucrase also catalyses very efficiently the elongation of glycogen branchings, an 80-fold increase in k_{cat} being observed in the presence of 10 g/L glycogen acceptor (*12*). Addition of acceptors greatly reduces the hydrolysis reaction and glucan synthesis, the major part of the glucosyl residues being transferred onto the acceptor.

Another interesting feature of glucansucrases is that their selectivities vary for each enzyme. A comparative study conducted with dextransucrase from *L. mesenteroides* NRRL B-512F and alternansucrase from *L. mesenteroides* NRRL B-1355 revealed that these enzymes display very different selectivities toward a sugar acceptors. For example, cellobiose, a poor acceptor for dextransucrase from *L. mesenteroides* NRRL B-512F, was found to be a fairly good acceptor for alternansucrase (*13*). As shown on Table II, series of oligosaccharides containing various types of glycosidic bonds α-1,2, α-1,3, α-1,6, β-1,4 were produced with alternansucrase wheras only product A was synthesized with B-512F dextransucrase.

Table II. Oligosaccharides produced from sucrose and cellobiose by alternansucrase from *L. mesenteroides* NRRL B-1355

Product	*Degree of polymerization*	*Structure*
A	3	α-D-Glcp-(1→2)-[β-D-Glcp-(1→4)]-D-Glcp
B	3	α-D-Glcp-(1→6)-cellobiose
C	4	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-cellobiose
D	5	α-D-Glcp-(1→3)-α-D-Glcp-(1→6)- α-D-Glcp-(1→6)-cellobiose
E	5	α-D-Glcp-(1→3)-[α-D-Glcp-(1→6)]- α-D-Glcp-(1→6)-cellobiose

These results indicate that the topology of the acceptor sites may be very different among glucansucrases and can probably be altered to modify the enzyme stereoselectivity and regioselectivity in order to increase the efficiency of glucosylation and control high molecular weight glucan or sucrose isomer synthesis. Therefore, the number of acceptors likely to be glucosylated by glucansucrases could probably be enlarged. In order to identify the glucansucrase structural determinants that define the specificity of each enzyme. For this purpose, it is necessary to get detailed structural information to determine the variations in glucansucrases that contribute to specificity.

Structural Data

Glucansucrase Classification

In the Carbohydrate Active Enzymes (CAZy) Database constructed on the basis of amino acid sequence similarities, enzymes with glucansucrase activity

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are found in two separate families: family 13 and family 70. Both families belong, with family 77, to the same clan GH-H of glycoside hydrolases and transglycosidases (*14*). Family 70 contains only enzymes acting on sucrose substrate. They all synthesize α-linked glucan polymers and catalyze tranglucosylation with retention of anomeric configuration. This family includes all the glucansucrases produced by lactic acid bacteria.

With more than 500 sequences and 25 different specificities, family 13 is a large group which contains hydrolases like α-amylases and transglycosidases like CGTase. Amylosucrase is the sole glucansucrase reported in this family. Enzymes from family 13 and family 70 share many common structural and mechanistic features (*15*). In particular, in both families, it is proposed that the reaction proceeds via a α-retaining double displacement mechanism which includes the following steps. First, a glutamic acid protonates the glycosidic oxygen. Simultaneously an aspartate residue exerts a nucleophilic attack on the C1 of the glucosyl ring, leading to the formation of an oxocarbonium ion-like transition state and then of a covalent intermediate. The departure of the aglycon part is followed by the entrance of an acceptor (water in the case of hydrolysis) which is activated by the deprotonated glutamic acid to attack the covalent bond between the C1 of the glucosyl ring and the aspartic acid. A third aspartate residue, always conserved in family 70 and family 13, would be involved in this mechanism ensuring the correct positioning of the glucosidic bond via a distortion of the glucosyl ring.

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Glucansucrases from Family 70

More than 30 homologous sequences of glucansucrases from *Leuconostoc*, *Streptococcus* or *Lactobacillus* genera have been reported but no 3D-structure is available yet. With an average molecular weight of about 160,000 Da, these large enzymes share a common primary structure organized in four regions: a signal peptide, a variable region in which only a few amino acids are conserved and a catalytic domain followed by a C-terminal glucan binding domain (Figure 1). The catalytic region is highly conserved and is proposed to possess a structure which resembles the characteristic $(\beta/\alpha)_8$ barrel of family 13 enzymes formed of eight β-strands (B) alternating with eight α-helices (H). This assumption is supported by the identification of short segments, along the catalytic domain of glucansucrases, identical to very conserved segments in family 13. Moreover, structure predictions indicates that glucansucrases possess a catalytic $(\beta/\alpha)_8$ barrel circularly permutated compared to α-amylase barrel. Indeed, the elements B1H1B2H2B3 are found behind the elements H3B4H4B5H5B6H6B7H7B8H8 along the sequence. These findings permitted the localization of highly conserved residues in both families (15). As expected, individual replacements of the three conserved carboxylic acids by their amide derivatives totally suppress the activity.

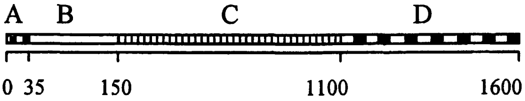

Figure 1. Schematic structure of glucansucrase A, N-terminal signal sequence; B, variable region; C, catalytic domain; D, glucan binding domain (GBD).

We have cloned several genes encoding glucansucrases. A first strategy based on the design of degenerated nucleotides from highly conserved sequences enabled the cloning of DSR-A, DSR-B (from *L. mesenteroides* NRRL B-1299), and DSR-C (from *L. mesenteroides* NRRL B-1355) (*16*). However this strategy was not successful for the cloning of the genes encoding the target enzymes *ie.* alternansucrase (ASR) and dextransucrase responsible for the synthesis of α-1,2 linkages (DSR-E). To isolate these genes, we proceeded first to micropurification and partial sequencing of the wild-type enzymes. Then, peptide sequences were used to construct probes specific for each enzyme. Using this method, *asr* and *dsr-e* encoding genes were successfully isolated (*17-18*).

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Comparison of DSR-S, DSR-E and ASR Sequences

DSR-S from *L. mesenteroides* NRRL-B-512 has a molecular mass of 175, 000. DSR-S primary structure, first determined by Wilke-Douglas *et al.* (*19*), is organized in four regions like all the glucansucrases from Family 70. In addition, highly conserved fragments located in the catalytic domain and shown on Figure 2 are also present in DSR-S. The three carboxylic acids presumably involved in the catalytic mechanism can be located at position 551, 588 and 662. Asp 551 was proposed to be the nucleophile. Accordingly, point mutation of this residue leads to a total loss of activity. Two other highly conserved residues, Asp511 and Asp513, were also changed into their amide derivatives (*16*). These residues had been proposed to be involved in sucrose binding. Mutations resulted in high or total loss of activity showing their importance.

Compared to all the other glucansucrases, ASR and DSR-E are remarkable, first by their unusual regiospecificities, but also by their molecular mass of 229 kDa and 313 kDa, respectively. To date, they are the largest glucansucrases reported. The size of alternansucrase is principally due to the length of the variable region and C-terminal domain. In general, ASR is organized like all the other glucansucrases but shows, all along the sequence, very distinctive features. First, sequence analysis revealed that some of the repeats usually found in the C-terminal domain of glucansucrases are found in the variable region of ASR. In

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GTFI	445	ANFDSIRV**D**AVDNVDADLLQI	486	HVSIV**E**AWSDND	557	YSFARAH**D**SEVQ**D**LI
GTFS	429	ANFDGVRV**D**AVDNVNADLLQI	470	HLSIL**E**AWSGND	540	YVFIRAH**D**SEVQ**T**RI
DSRS	543	ANFDGIRV**D**AVDNVDADLLQI	584	HLSIL**E**DWSHND	655	YSFVRAH**D**SEVQ**T**VI
DSRB	525	ANFDGIRV**D**AVDNVDADLLQI	566	HLSIL**E**DWSHND	637	YSFVRAH**D**SEVQ**T**VI
ASR	626	ANFDGIRV**D**AVDNVDADLLKI	667	HLSIL**E**DWNGKD	759	YSFVRAH**D**YDAQ**D**PI
C_1	519	ANFDGYRV**D**AVDNVDADLLQI	560	HISIL**E**DWDNND	631	YAFIRAH**D**SEVQ**T**VI
C_2	2202	ANFDSIRI**D**AVD***FIHN***D***TIQR***	2243	HISLV**E**AG***LDAG***	2315	YSIIHAH**D*****KGVQEKV***
		◆		●		Δ

Figure 2. Alignment of highly conserved sequences in glucansucrase catalytic domains: GTFI, S. downei Mfe28; GTFS, S. downei Mfe2; DSRS, L. mesenteroides NRRL B-512F ; DSRB, L. mesenteroides NRRL B-1299 ; ASR, L. mesenteroides NRRL B-1355; C_1 and C_2, catalytic domains of DSR-E, L. mesenteroides NRRL B-1299. ◆ putative nucleophile; ● putative general acid catalyst; Δ aspartic acid involved in distorsion of glucosyl ring; Underlined fragment corresponds to sequences diverging from consensus ones

addition, the enzyme contains new repeats in the C-terminal domain. These new motifs don't play a crucial role in the enzyme activity and specificity. Indeed, ASR deleted of its C-terminal domain displays the same specificity and is still highly active compared to the wild type enzyme.

Finally, DSR-E structure is even more original. Indeed, this huge enzyme is formed by two catalytic domains of about 900 amino acids separated by a long glucan binding domain (Figure 3). Both domains are homologuous to the usual N-catalytic domain of glucansucrases. The highly conserved blocks of family 70 which contain amino acids presumed to participate in catalysis are easily identified in ASR catalytic domain and in the two second catalytic domains of DSR-E. In particular, it is possible to locate easily the three putative carboxylic acids in each of the catalytic domains of DSR-E suggesting that both domains are active. In addition, both ASR and the second domain of DSR-E possess distinctive stretches of sequences in usually highly conserved regions (in the vicinity of the putative carboxylic residues) (Figure 2). Although it is not possible, without structural data, to clearly determine the role of these residues, it is likely that they influence substrate or acceptor placement in the active site. These residues are thus good candidates for site-directed experiments.

Of course, the presence of two catalytic domains raises the question of the contribution of each independent domain to the catalysis and specificity of DSR-E. Deletion experiments were carried out to elucidate this point. They showed that each independent domain is active and further suggest that the first domain would be responsible for the synthesis of α-1,6 linkages whereas the second one would be the only one responsible for α-1,2 specificity. These results now deserve a confirmation by site-directed mutagenesis. The role of the GBD in this very complex machinery will also be investigated.

Information issued from the sequence analysis of the very uncommon enzymes DSR-E and ASR enabled the identification of amino acids and motifs putatively associated with specificity. Further investigations are needed to confirm the functional or structural role of these candidates. Selectivity and specificity of these enzymes surely involve many different determinants and the lack of 3D-structure undoubtedly limits the structure function studies. Given this fact, it is crucial to exploit the relatedness between family 13 and 70 and especially the information issued from the recent 3D-structure determination of amylosucrase.

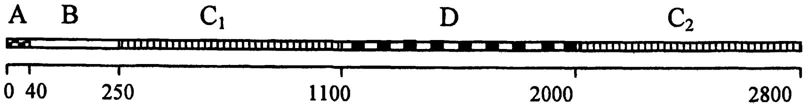

Figure 3. Schematic structure of DSR-E
A, N-terminal signal sequence; B, variable region; C_1 and C_2 catalytic domains; D, glucan binding domain (GBD).

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Glucansucrase from Family 13 : the Case of Amylosucrase

Amylosucrase from *N. polysaccharea* is the only glucansucrase found in family 13. At present, it is the only glucansucrase for which a three dimensional structure has been reported. This enzyme, at the boundary between α-amylases and glucansucrases, was cloned, sequenced and purified to homogeneity for structural and biochemical characterization. The crystal structure of amylosucrase, determined to 1.4 Å resolution by Skov *et al. (20)*, revealed that the 636 amino acids of amylosucrase are arranged in five domains as shown in Figure 4.

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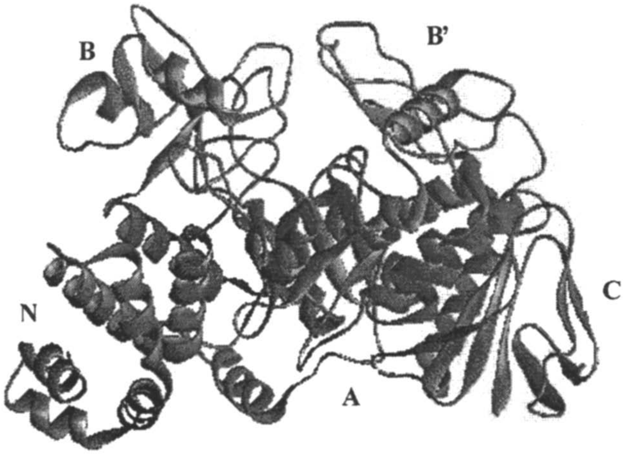

Figure 4. Schematic representation of the AS structure with the five domains N, A, B, B' and C

First, the N terminal domain shows a structure never identified in any other protein. It is composed of six amphiphilic helices and two of which interact with the following domain A. This latter consists of a central catalytic $(\beta/\alpha)_8$ barrel domain, a common characteristic of all enzymes from family 13. Two loops are emerging from this domain and are long enough to be labeled as domains. Loop 3 between β-strand 3 and α-helix 3 corresponds to the B-domain also found in most of the members of family 13. Loop 7, also called domain B', ensures the junction between β-strand 7 and α-helix 7 and is a unique feature of amylosucrase. Finally, domain C (residues 555-628), also found in many other α-amylases , consists of an eight-stranded β-sheet domain at the C-terminal end of the protein. Amylosucrase has a lot of structural similarities with the oligo-1,6-glucosidase (an exo-acting enzyme specific for the cleavage of α-1,6 glucosidic bond) and TAKA-amylase. In TAKA-amylase, several residues around subsite −1 (according to the nomenclature proposed by Davies et al., *21*) are known to be involved in the catalytic mechanism. In amylosucrase, these residues (Glu 328, Asp 286, Asp393 and His 187 and His 392) occupy identical positions. This observation is in agreement with a double deplacement mechanism similar to the one observed for α-amylases. Glu 328 is proposed to act as the general catalyst and Asp 286 as the nucleophile *(20-21)*.

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Understanding the sucrose specificity

The amylosucrase active site ressembles a pocket covered by domain B and domain B' (loop 7). The X-ray structure of the active site Glu328Gln amylosucrase mutant in complex with sucrose revealed that sucrose is bound at the bottom of this pocket with the glucosyl ring at the −1 position and the fructosyl ring at sub-site +1 (*22*). In the subsite numbering established by Davies et al., the cleavage of the glycosidic bond occurs between subsite −1 and subsite +1. The glucosyl ring of sucrose is bound by a network of hydrogen bonds involving the five conserved residues at subsite −1 and hydrophobic interactions with the aromatic residues Tyr 147 and Phe250. In addition, a salt bridge between Asp 144 and Arg 509 blocks the bottom of the pocket (Figure 5). These residues are not conserved in family 13 and interact by hydrogen bonding with *O*4 of sucrose glucosyl ring. Point mutations of these residues resulted in a complete loss of activity showing that they presumably play a major role in the specificity of amylosucrase for sucrose. Amylosucrase interacts more weakly with the fructosyl ring via four direct and four water mediated hydrogen bonds. These interactions are presumed to ensure a correct positioning of the lone pair of the linking *O*1 for hydrogen bonding with general acid catalyst Glu328 facilitating a nucleophilic attack by Asp286.

A Possible Mechanism for Polymer Synthesis

Recently, an X-ray structure of Glu328Gln amylosucrase mutant in complex with maltoheptaose revealed three maltoheptaose binding sites: OB1 which corresponds to the active site, OB2 close to the active site on the surface of the B' domain and OB3 on the surface of the C-terminal domain (Figure 6) (*23*).

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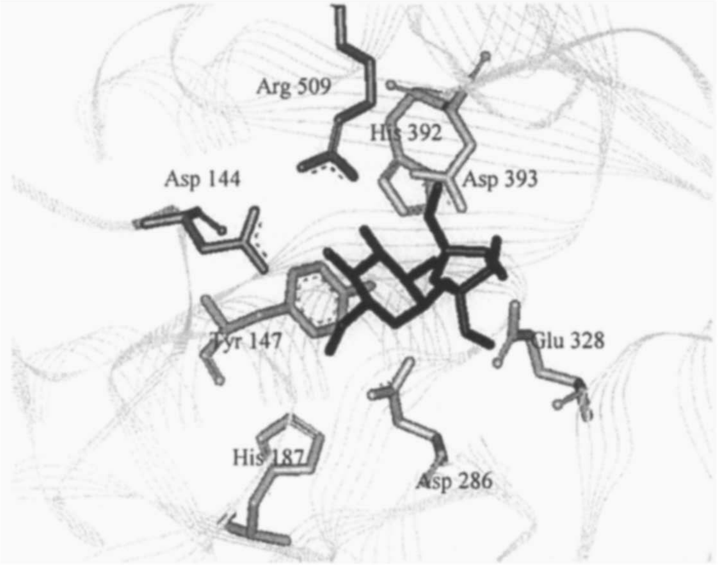

Figure 5. Sucrose molecule in the active site of amylosucrase

Maltoheptaose binds in the active site with the non-reducing end positioned in sub-site −1. This is in perfect agreement with the disproportionation reaction of maltooligosaccharides catalyzed by amylosucrase in which amylosucrase transfers one glucosyl unit from the non-reducing of a maltooligosaccharide donor to the non–reducing end of a maltooligosaccharide acceptor (*24*). The presence of maltoheptaose in the active site gives also a good representation of what happens after the formation of the glucosyl-enzyme. X-ray analysis of the complex enabled the mapping of the acceptor binding site upto subsite +5. Site-directed mutagenesis experiments are under progress to confirm the role of the residues involved in acceptor binding. The second binding site (OB2) at the surface of B' domain is a unique feature of amylosucrase. B' domain doesn't exist in other enzymes from family 13 and is thought to be highly involved in the polymerase specificity of amylosucrase. In a previous study, we observed during the course of polymer synthesis the production of significant amounts of glucose, maltose, maltotriose and sucrose isomers (turanose, and trehalulose) but did not detect maltooligosaccharides of longer size (*25*).

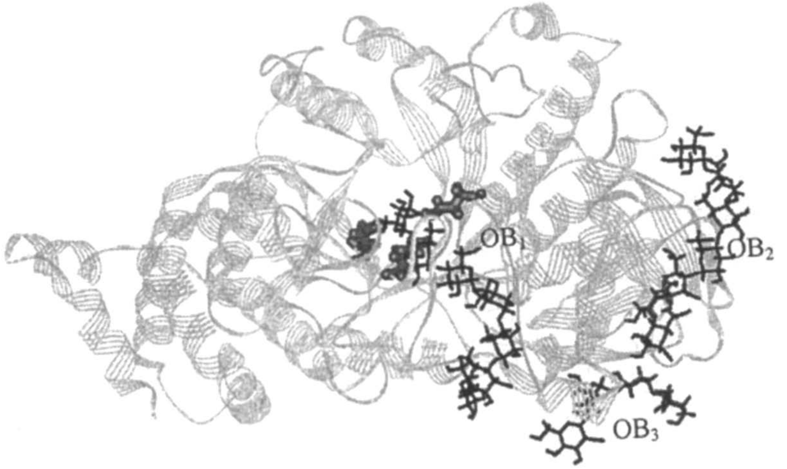

Figure 6. Glu328Gln amylosucrase mutant in complex with maltoheptaose Light grey, amylosucrase backbone; Dark grey residues, active site carboxylic acids; black; maltoheptaose molecules.

We suggested that the polymer synthesis was processive. However, by increasing the sensitivity of the detection methods, longer maltooligosaccharides from DP4 to 20 were identified in the medium. The concentration of these products is very low. However, their presence suggests that elongation occurs at the non-reducing end and that the mode of polymer synthesis is sequential. During the reaction, the concentration of oligosaccharides of DP higher than 3 is maintained at a very low level. The enyme prefers to elongate the longer oligosaccharides for which the affinity probably increases with size. This behaviour can be correlated to the presence of OB2, which could be eventually connected to OB1, to ensure high affinity binding sites for oligosaccharides of increasing size. Finally, the equilibrium would be shifted toward polymer synthesis because of the insolubility of the amylose chain. Further biochemical and structural investigations are still necessary to fully elucidate the mode of action of amylosucrase. However, due to the similarity existing between amylosucrase and glucansucrases from family 70, our recent findings will certainly help for the understanding of the family 70 glucansucrase mechanism, which is still a matter of debate.

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New Perspectives for Glucansucrase Engineering

Regarding amylosucrase, the route for rational design is now opened. Following structural analysis, site-directed experiments have started already to modulate the activity of the enzyme. In addition, modification of the acceptor selectivity can be envisaged by using information from molecular modelling to increase the affinity of the less efficient acceptors via site-directed mutagenesis. Concerning glucansucrases with a permutated barrel, information issued from sequence analysis served to identify regions probably involved in the specificity of these enzymes and mutagenesis experiments should confirm the implication of the target residues. However, the drawback is the absence of three dimensional structures. In addition, the outcome of a mutation rarely corresponds to what was expected because of the enzyme complexity. For all these reasons, *in vitro* evolution using error-prone PCR in combination with DNA shuffling must be considered to evolve glucansucrases. The advantage of such approaches is that three dimensional structures are not required. Moreover, *in vitro* evolution has been demonstrated to be very efficient to improve thermostability or efficiency of naturally occurring enzymes by mutations of amino acids that cannot be identified from structural analysis. We are just beginning to experiment with these methods to improve the catalytic efficiency and stability of amylosucrase and will hopefully extend this work very soon to other glucansucrases.

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Chapter 9

Kojioligosaccharides: Application of Kojibiose Phosphorylase on the Formation of Various Kojioligosaccharides

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Various kojioligosaccharides such as kojibiose, kojitriose, kojitetraose, 2-*O*-α-kojibiosyl-β-D-fructofuranoside, 1-*O*-α-kojibiosyl-α-D-glucopyranoside, and 4-*O*-α-kojibiosyl-D-glucose were enzymatically synthesized using kojibiose phosphorylase (KPase) from a thermophilic anaerobe, *Thermoanaerobacter brockii.* Combination of KPase with other phosphorylases, such as maltose phosphorylase or trehalose phosphorylase, has potential applications in production of novel saccharides from inexpensive sugars. Structures and functions of kojioligosaccharides are also presented.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Kojibiose (2-*O*-α-D-glucopyranosyl α-D-glucopyranose) is a disaccharide that occurs in koji extract *(1)*, beer, honey *(2)*, and starch hydrolyzate. The best known method for preparation of kojibiose is the isolation from a partial acetolyzate of dextran from *Leuconostoc mecenteroides* NRRL B-1299 *(3)*, although the method is obviously a tedious one. Although many researchers have attempted to synthesize kojibiose using glycosidases such as α-glucosidase *(4)*, glucoamylase *(5)* or sucrose phosphorylase *(6)*, there are some problems such as the formation of by-products and a low efficiency for production. On the other hand, kojioligosaccharides of DP3 or higher, such as kojitriose, kojitetraose and kojipentaose, are rare in nature. Therefore, these have been prepared by chemical synthesis *(7-8)*.

We isolated a novel enzyme, kojibiose phosphorylase (KPase) from a thermophilic anaerobe, *Thermoanaerobacter brockii* ATCC 35047 *(9)*. This enzyme catalyzes the reversible phosphorolysis of kojibiose. In the presence of suitable acceptors such as mono- or oligo-saccharides, KPase also catalyzes α-1,2-glucosyl transfer from β-D-glucose-1-phosphate (β-G1P) to the acceptors *(10)*, and produces various kojioligosaccharides *(11-12)*.

This paper describes the properties of KPase, the enzymatic synthesis of various kojioligosaccharides, and on some properties and functions of these sugars.

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Properties of Thermoanaerobacter Kpase

Properties of Thermoanaerobacter KPase

We have previously reported on the purification and characterization of KPase (10). Properties and the deduced amino acid sequence of the enzyme (13) are shown in Table I and Figure 1, respectively. The apparent molecular mass of KPase was estimated to be 500 kDa by gel filtration. On the other hand, SDS-PAGE of the purified KPase gave a single protein band with an apparent molecular mass of 80 kDa. These results suggest that the enzyme consists of six identical subunits. The optimum pH was 5.5 for both the phosphorolytic and the synthetic reactions. Maximum activity was observed at 65°C. The enzyme was stable from pH 5.5 to 9.7, and at temperatures up to 65°C.

A KPase gene has been cloned from *Thermonanaerobacter brockii* ATCC 35047 and sequenced to obtain the amino acid sequence of KPase. KPase is composed of 755 amino acid residues, and its molecular mass is calculated to be about 90 kDa. The putative three catalytic amino acid residues, aspartate-362, lysine-614 and glutamate-642 were deduced by site-directed mutational analysis (data not shown).

Table I. **Enzymatic properties of KPase from *Thermoanaerobacter brockii.***

Molecular mass (Da)	
SDS-PAGE	83,000
Gel filtration	500,000
Isoelecric point	4.3
Optimum pH[a]	5.5
Optimum temperature (°C)[b]	65
pH Stability[c]	5.5 – 9.7
Thermal stability (°C)[d]	below 65

[a], Both phosphorolytic and synthetic reaction; [b], 30-min reaction; [c], after 20-hr incubation at 4°C; [d], after 60-min incubation.

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MVKHMFLEDV NNLISDDKWL IFQNEYNTEV NPRYETLFTL TNGYMGVRGT  50
FEEGSEGERS GNFIAGIFDK SDAQVREIVN AQNWLRIKLY VEGEELSLDK 100
CQLIEFKRIL DMKKGILFRS MLIKDSKDRI TRIEGYRFIS RSDLHRSAIK 150
LFVTPVNYSG VVGIESIIDG TVLNSADSPK HRVKHLKVAD NSSLNKSGVY 200
LETATIDDDI RIATGSAVRL YHYEDKEKNN IAKFKRFLPL GEMSIEYFEF 250
DGTENKTVVI DKFIITYTSR DVKKGLLKST VEKELFAFAG EGIDKELQRH 300
IEVYEELWSV ADINIEGDEE ADKALRFNIF HLMSSVNEND PMVSIAAKAL 350
HGEGYKGHVF WDTEIFMLPF FIYVHPKAAK TLLMYRYNML DAARKNAALN 400
GYKGAQYPWE SADTGEEETP KWGFDYMGNP VRIWTGDLEH HITADIAFAV 450
WEYFRATEDI EFMLNYGAEV IFETARFWVS RCEYVKELDR YEINNVIGPD 500
EFHEHVDNNA YTDYLAKWNI KKGLELINML KEKYPEHYHA ISNKKCLTNE 550
EMEKWKEVEE KIYIPYDKDK KLIEQFEGYF DKKDYVIDKF DENNMPIWPE 600
GVDITKLGDT QLIKQADVVM LMLLLGEEFD EETKRINYEY YEKRTMHKSS 650
LGPSMYAIMG LKVGDHKNAY QSFMRSANVD LVDNQGNTKE GLHAASAGGT 700
WQVVVFGFGG MEIDKEGALN INSWLPEKWD KLSYKVFWKG NLIEVIVTKQ 750
EVTVKKLKGK GNIKVKVKGK ELTIE                       775
```

Figure 1. Amino acid sequence of KPase from Thermoanaerobacter brockii.

The underlined amino acid sequences are those found from protease digestion products of KPase protein. The open-boxed amino acids are the putative three catalytic residues deduced from site directed mutational analysis.

Substrate and Acceptor Specificities of KPase

KPase was specifically active on kojibiose and inactive on other disaccharides such as sophorose, trehalose, neotrehalose, nigerose, laminaribiose, maltose, cellobiose, isomaltose, gentiobiose, sucrose and lactose (data not shown). Acceptor specificity was examined using β-G1P sodium salt as a glucosyl donor and various mono-, di-, and oligo-saccharides as acceptors. As shown in Table II, D-glucose, L-sorbose, methyl-α-D-glucopyranoside, and methyl-β-D-glucopyranoside were effective acceptors among various monosaccharides. D- and L-Xylose also acted as acceptors, but their transfer ratio was less than 25%. Furthermore, maltose, trehalose, nigerose, isomaltose, maltotriose, and others were good oligosaccharide acceptors. These results suggest that di- and oligosaccharides having a D-glucosyl residue at their non-reducing ends were good acceptors in any case.

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Table II. Acceptor Specificity of KPase.

Acceptor	*Product*	*Acceptor*	*Product*
D-Glucose	+++	α,α–Trehalose	+++
D-Xylose	+	Neotrehalose	+++
L-Xylose	+	Kojibiose	+++
D-Galactose	-	Nigerose	+++
D-fructose	-	Maltose	+++
D-Mannose	-	Isomaltose	+++
D-Arabinose	-	Laminaribiose	+++
D-Fucose	-	Cellobiose	++
L-Fucose	-	Gentiobiose	++
L-Sorbose	+++	Maltitol	+++
D-Ribose	-	Sucrose	++
L-Rhamnose	-	Palatinose	+++
Methyl-α-D-glucoside	+++	Maltulose	+++
Methy-β-D-glucoside	+++	Turanose	+++
2-Deoxy-D-glucose	-	Lactose	-
N-Acetyl-D-glucosamine	-	Melibiose	-
D-Glucosamine	-	Lactulose	-
Sorbitol	-	Maltotriose	+++
		Maltotetraose[a]	+++
		Maltopentaose[a]	+++

The transfer ratio to acceptor was detected by GLC. +++, 50%<transfer ratio; ++, 25%<transfer ratio<50%; +, 0%<transfer ratio<25%; -, no detected transfer product to acceptor. [a]Detected by TLC.

Enzymatic Syntheses of Various Kojioligosaccharides

Formation of Kojioligosaccharides from β-G1P and D-Glucose

We examined the formation of kojioligosaccharides linked with only α-1,2 glucosidic linkages from β-G1P and D-glucose. 100 mM of D-glucose and 200 mM β-G1P were subjected to KPase. HPLC profile of the reaction mixture is shown in Figure 2. Oligosaccharides of DP2 to DP6 were detected. The percents of DP2, DP3, and DP4 were 23.5%, 35.9%, and 10.1%, respectively. These three oligosaccharides were purified up to 92% purity by gel permeation chromatography. The yield for the di-, tri- and tetra-saccharides were approximately 26, 34, and 4.5 grams, respectively. Using methylation and ^{13}C-NMR analyses, we identified these oligosaccharides as kojibiose (*O*-α-D-glucopyranosyl-(1→2)-D-glucopyranose), kojitriose (*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose) and kojitetraose (*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose).

A reaction mixture (6L) containing 312 g of β-G1P, 108 g of D-glucose and 3120 U of KPase was incubated at pH 5.5 and at 60°C for 24 hr. After stopping

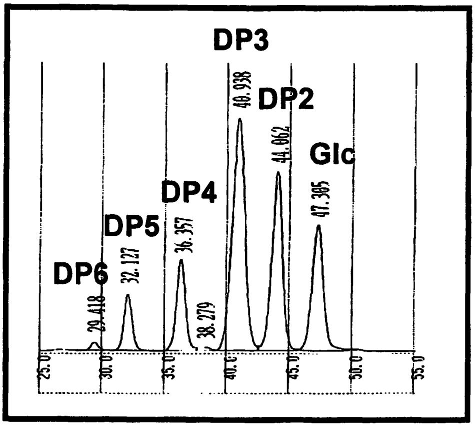

Figure 2. HPLC chromatogram of kojioligosaccharides synthesized from β-G1P and D-glucose using KPase.

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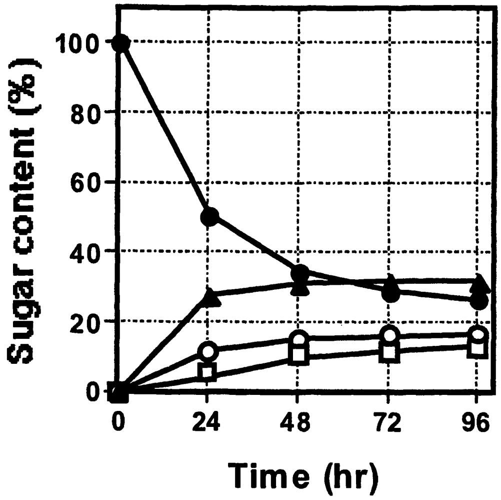

Figure 3. Formation of 1-O-α-kojibiosyl-α-D-glucopyranoside (selaginose) from α,α–trehalose by TPase and KPase.The reaction mixture (final volume:10 ml) containing 5% (w/w) α,α–trehalose, 20 mM acetate buffer (pH5.5), 2 mM sodium phosphate, 2 U of TPase, and 2 U of KPase was incubated at 60°C for 96 hr. Aliquots (1 ml) were withdrawn at various intervals, and the sugar composition in the reaction mixture was determined by HPLC.
●, α,α–trehalose; ▲, selaginose; ○, D-glucose; □, kojibiose.

the reaction by boiling for 30 min, sugar composition in the reaction mixture was analyzed by HLPC using MCI-gel CK04SS column.

Formation of 1-*O*-α-Kojibiosyl-α-D-glucopyranoside (Selaginose) from α,α–Trehalose by Combination of KPase and TPase

A trisaccharide was enzymatically synthesized from α,α-trehalose by simultaneous reaction of TPase (14) and KPase. The scheme of the reaction is as follows: Trehalose + Pi —(TPase)→ D-glucose + β-G1P, β-G1P + trehalose —(KPase)→ glucosyl-trehalose + Pi. The time course of the reaction is shown in Figure 3. The main product was a non-reducing trisaccharide, which was produced in a yield of 32%. The trisaccharide was easily isolated, using HPLC followed by alkaline-treatment (pH 12, 100°C for 90 min) which specifically decomposes reducing sugars. The structure of the isolated trisaccharide was determined to be *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1↔1)-α-glucopyranoside (1-*O*-α-kojibiosyl-α-D-glucopyranoside). It has been reported that this tri-saccharide occurs in a plant, *Selaginella,* and has been known by the name of "selaginose" *(15-16).*

Formation of 4-*O*-α-Kojibiosyl-D-Glucose

Formation of kojioligosaccharides from maltose was examined using a simultaneous reaction of KPase and maltose phosphorylase (MPase). MPase was prepared from cytoplasmic fraction of *Enterococcus hirae* IFO 3181 *(17).*

Figure 4 shows the time courses of the reaction under optimal conditions. As shown in Figure 4, 4-*O*-α-kojibiosyl-D-glucose increased to 35.6% at the early stage (2 hr) of the reaction. However thereafter, the sugar decreased, while kojibiose and other kojioligosaccharides increased. Total sugar content of kojioligosaccharides was 68.5 % after reaction for 48 hr.

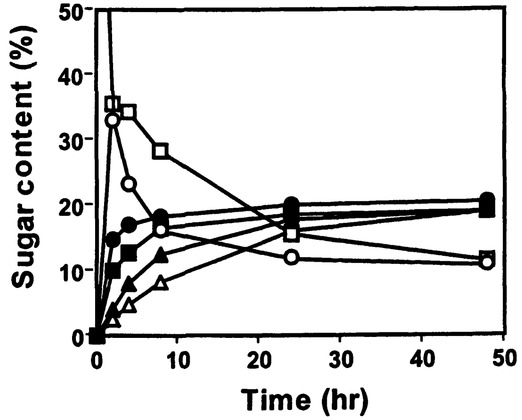

Figure 4. Formation of kojioligosaccharides from maltose by MPase and KPase. A reaction mixture (3 ml) containing 200 mM of maltose, KPase (3.4 U/mmol-maltose), MPase (3.4 U/mmol-maltose) and 10 mM sodium phosphate was incubated at pH 6.0 and at 50°C for 48 hr. ○*, maltose;* □*, 4-O-α-kojibiosyl-D-glucose;* ●*, D-glucose;* ■*, kojibiose;* ▲*, kojitriose;* Δ*, others (DP>3)*

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Formation of 2-*O*-α-Kojibiosyl-β-D-Fructofuranoside

A glucosyl-sucrose was prepared by KPase using β-G1P as a D-glucosyl donor and sucrose as an acceptor. The β-G1P:sucrose molar ratio was set at 1:2 so as to increase the yield of glucosyl-sucrose.

A reaction mixture (400 ml) containing 200 mM of β-G1P, 400 mM sucrose and KPase (5 U/g-G1P) was incubated at pH 5.5 and at 60°C for 48 hr. As shown in Table III, the content of glucosyl-sucrose reached to 33.6% at maximum.

Table. III. Preparation of glucosyl-sucrose by KPase.

Sugar composition (%)			
β-G1P	*Suc*	*G-Suc*	*Others (DP4)*
9.3	52.5	33.6	4.6

Suc, sucrose; G-Suc, glucosyl-sucrose;

The glucosyl-sucrose in the reaction mixture was purified up to 97%-purity by Toyopearl HW-40S gel permeation choromatography. The structure of the sugar was determined to be *O*-α-D-glucopyranosyl-(1 → 2)-*O*-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside (2-*O*-α-kojibiosyl-β-D-fructofuranoside).

The structural formulas of kojioligosaccharides that were synthesized are shown in Figure 5.

Properties and Functions of Kojioligosaccharides

Reducing Power and Maillard Reaction of Kojibiose, Kojitriose and Kojitetraose

Compared with glucose, the reducing powers of kojioligosaccharides was measured by Nelson-Somogyi method (18). Although kojioligosaccharides are reducing sugars, having an aldehyde group at C-1 position of the end glucose, the reducing powers of kojibiose, kojitriose and kojitetraose were much less than 1% of that of glucose (data not shown).

As shown in Figure 6, their low reducing power weakens the Maillard reaction of kojioligosaccharides with amino acids. Further, kojioligosaccharides

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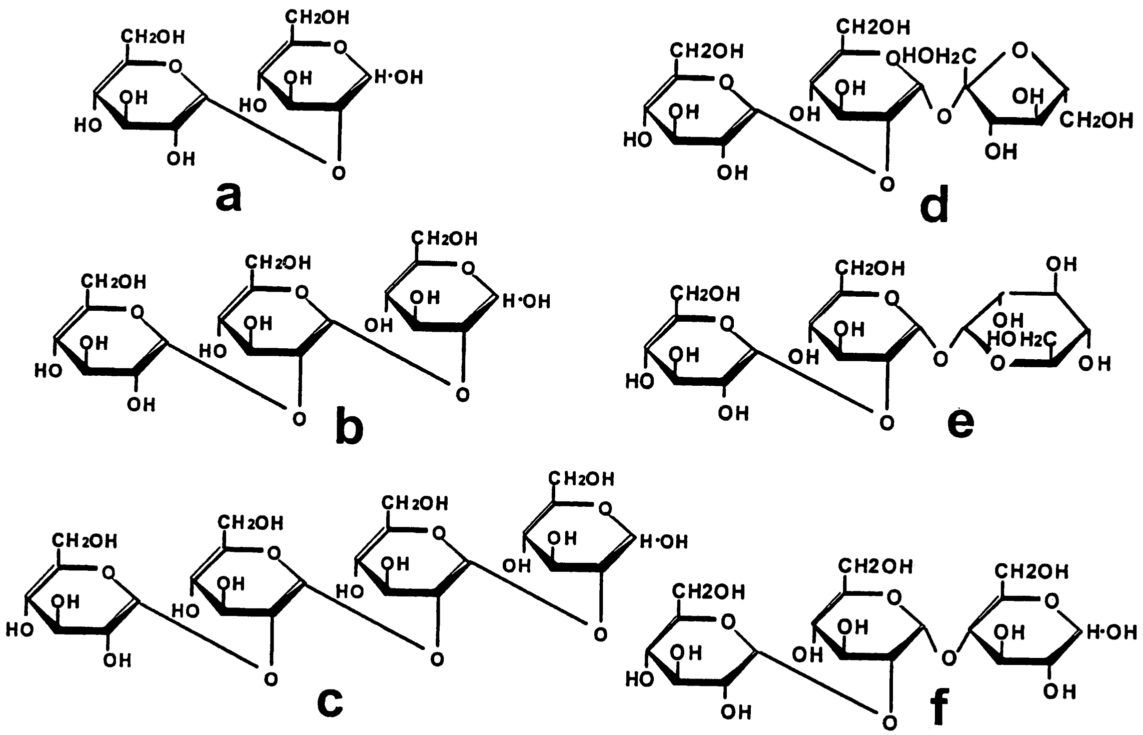

Figure 5. Several kojioligosaccharides synthesized using KPase. a, kojibiose; b, kojitriose; c, kojitetraose; d, 2-O-α-kojibiosyl-β-D-fructofuranoside; e, 1-O-α-kojibiosyl-α-D-glucopyranoside; f, 4-O-α-kojibiosyl-D-glucopyranose.

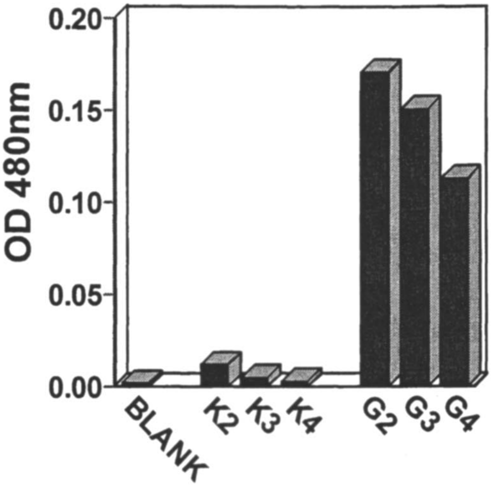

Figure 6. Maillard reaction of kojioligosaccharides. Equal volume of 5% sugar solution and 1% glycine solution were mixed and incubated at pH 8.0, 100°C for 90 min. K2, kojibiose; K3, kojitriose; K4, kojitetraose; G2, maltose; G3, maltotriose; G4, maltotetraose

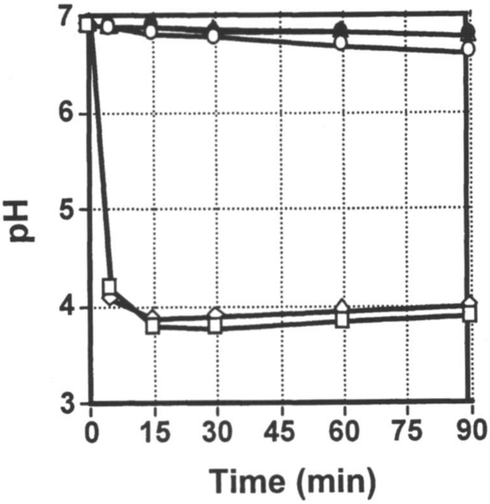

Figure 7. Acid-formation by a cariogenic bacterium, Streptococcus mutans OMZ-176. Equal volume of 50 % of S. mutans cell suspension and 1% sugar solutions were mixed and incubated at 37°C for 90 min. Changes of pH levels were measured at various intervals. □, sucrose; ◊, maltose; ○, kojibiose; ●, kojitriose; Δ, kojitetraose.

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have a mild amount of sweetness. These features should make kojioligosaccharides attractive materials in the food industry.

Acid Formation by *Streptococcus mutans*

Acid-formation by *Streptococcus mutans* from kojioligosaccharides was examined (Figure 7). It is well known that *Streptococcus mutans* causes dental caries. When sucrose is ingested, the bacterium liberates glucosyltransferase to synthesize both adhesive and non-adhesive glucans from sucrose. Adhesive glucan is the major cause of dental plaque because the bacterium lives in it, and converts sugars into organic acids, which degrade the enamel of teeth. Sucrose and maltose decreased pH level, while kojioligosaccharides did not. These results may suggest that kojioligosaccharides are not utilized and converted into adhesive glucan and acids by the bacterium.

Kojioligosaccharides do not inhibit the formation of adhesive glucan from sucrose (data not shown). Sugars can be classified into three types: cariogenic, non-cariogenic, and cariostatic types. According to this classification, kojioligosaccharides are of non-cariogenic type.

In vitro Digestibility of Kojioligosaccharides

In order to investigate digestibility of kojioligosaccharides, *in vitro* digestion tests were carried out using human saliva, artificial gastric juice, porcine pancreatic amylase and rat intestinal enzyme according to the method of Okada *et al. (19)*. Maltose was used as a control.

No hydrolysis of maltose, kojibiose, kojitriose, kojitetraose, 4-*O*-α-kojibiosyl-D-glucose, or 2-*O*-α-kojibiosyl-β-D-fructofuranoside was observed using salivary, artificial gastric juice or pancreatic amylases. Maltose and kojibiose were hydrolyzed by small intestinal enzymes, while kojitriose, kojitetraose, 2-*O*-α-kojibiosyl-β-D-fructofuranoside and 4-*O*-α-kojibiosyl-D-glucose, were not hydrolyzed (Table IV). These results suggest that almost all ingested kojibiose is digested and absorbed in the small intestine, as well as kojioligosaccharides of DP3 or higher that are brought into the large intestine and are neither digested nor adsorbed in the small intestine.

Utilization by Intestinal Bacteria

Utilization of kojioligosaccharides by bacteria of the large intestine was examined, using 22 strains of 8 distinct genera. As shown in Table V, 12 strains out of 22 intestinal bacteria tested utilize kojibiose for their growth. Kojitriose and kojitetraose were utilized by a few bacteria, such as *Bifidobacterium breve*,

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Table IV. *In vitro* Digestibility of Kojioligosaccharides

	Hydrolysis (%)					
	G2	*K2*	*K3*	*K4*	*KF*	*KG*
Human saliva	0.0	0.0	0.0	0.0	0.0	0.0
Artficial gastric juice	0.0	0.0	0.0	0.0	0.0	0.0
Porcine pancreas	0.0	0.0	0.0	0.0	0.0	0.0
Rat intestinal acetone powder	87.5	56.8	0.0	0.0	0.0	0.0

Digestion tests were carried out according to the method of Okada *et al.*(19)

G2, maltose; K2, kojibiose; K3, kojitriose; K4, kojitetraose; KF, 2-*O*-α-kojibiosyl-β-D-fructofuranoside; KG, 4-*O*-α-kojibiosyl-D-glucose.

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Eubacterium aerofaciens and *Peptostreptococcus productus*. These results may suggest that kojioligosaccharides of DP3 or higher would find uses as low calorie sugars.

Conclusion

We isolated a novel enzyme, kojibiose phosphorylase (KPase) from a thermophilic anaerobe, *Thermoanaerobacter brockii*. This enzyme is useful to synthesize various kojioligosaccharides. Combination of KPase with other phosphorylases, such as maltose phosphorylase or trehalose phosphorylase, has potential applications in production of novel saccharides from inexpensive sugars. Kojibiose, kojitriose and kojitetraose have weak reducing power. This low reducing power weakens the Maillard reaction of these sugars. It is suggested that kojioligosaccharides, except kojibiose, are not readily hydrolyzed when ingested. It is possible that these sugars could be low calorie sugars. Studies on the function and usefulness of kojioligosaccharides are in progress.

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Table V. Utilization of Kojioligosaccharides by Intestinal Bacteria.

Bacterial species	*G1*	*K2*	*K3*	*K4*
Bifidobacterium				
B. adolecentis JCM1275	+++	-	-	-
B. bifidum JCM1255	++	-	-	-
B. breve JCM1192	+++	+++	+++	+++
B. infantis JCM1222	+++	+	-	-
B. longum JCM1217	+++	+	-	-
Lactobacillus				
L. acidophilus JCM1132	++	-	-	-
L. gasseri JCM1131	+++	+++	-	-
L. reuteri JCM1112	++	-	-	-
L. salivarius JCM1231	+++	+++	-	-
Eubacterium				
E. limosum JCM6421	+++	-	-	-
E. aerofaciens ATCC25986	+++	+++	++	+
Bacteroides				
B. distasonis JCM5825	++	+	-	-
B. vulgatus JCM5826	+++	++	-	-
B. ovatus JCM5824	+++	-	-	-
Clostridium				
C. butyricum JCM1391	+++	+++	-	-
C. perfringens JCM3816	++	+++	-	-
C. ramosum JCM1298	++	+++	-	-
C. paraputrificum JCM1293	+++	-	-	-
Streptococcus				
S. faecalis IAM10065	+++	-	-	-
Peptostreptococcus				
P. prevotii ATCC9321	+++	-	-	-
P. productus ATCC27340	++	+++	+++	+++
Escherichia				
E. coli IFO3301	+	-	-	-

Utilization test was carried out according to the method of Mitsuoka *et al.* *(20)*, using glucose as control. Each bacterium was inoculated into a culture medium, containing either of the sugars, and cultivated at 37°C for 4 days. Utilization of sugars were estimated by measuring pH levels of resulting culture broth. +++, < pH 4.9; ++, pH 5.0 - pH 5.4; +, pH 5.5 – pH 5.9; -, pH 6.0 <.

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Chapter 10

Medical Foods and Fructooligosaccharides: A Novel Fermentable Dietary Fiber

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Short chain fructooligosaccharides (scFOS) consist of two to four D-fructofuranosyl units linked by β-(2→1) glycosidic bonds and carry a single α-D-glucopyranosyl unit at the non-reducing end of the chain, linked (1↔2) as in sucrose. Because of their low molecular weight, scFOS are not quantified as total dietary fiber. However, this attribute makes them compatible with liquid medical foods, many of which are fed to patients through a tube. Most sources of dietary fiber are not compatible with liquid medical foods: insoluble fibers tend to settle and can block the feeding tube, whereas soluble fibers increase product viscosity making it difficult to administer through an enteral tube. Short chain FOS have many dietary fiber-like physiological effects. Medical rationales for their use include: normalizing bowel function, maintaining large bowel integrity, restoring colonization resistance, altering the route of nitrogen excretion, and improving calcium absorption. Overall, compatibility with liquid products and numerous physiological benefits to the patient justify the use of scFOS in medical foods.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

Numerous health benefits are associated with the consumption of dietary fiber. Dietary fiber may provide bulk to the stool, decrease intestinal transit time (e.g., relieve constipation), attenuate glycemic response, improve cholesterol and lipid metabolism, and reduce the risk of colon cancer. The Life Sciences Research Office, Federation of American Societies for Experimental Biology (*1*) recommends that we consume between 20 to 35 g of total dietary fiber daily of which 70 to 75% should be insoluble and 25 to 30% soluble. Most individuals can meet this recommendation by incorporating grains, fruits, and vegetables into their diet. However, certain individuals must use a medical food to meet their dietary needs. A medical food is defined by the U.S. Food and Drug Administration (*2*) as "a food that is formulated to be consumed or administered enterally under the supervision of a physician and is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation." Medical foods are typically liquid because patients may require that the product be administered via an enteral feeding tube. Internal diameter of an enteral feeding tube, for obvious reasons, is small. If a medical food is fed without the aid of a pump, its viscosity must be kept below 100 millipascals/sec (i.e., 100 centipoise). Unfortunately, dietary fiber, particularly soluble dietary fiber, has a tendency to increase the viscosity of liquid medical foods. Also, insoluble fibers settle to the bottom of the container, increasing the risk of tube clogging. Hence, it was imperative that sources of fiber or fiber-like material be identified that provided physiological benefits but did not compromise the physical stability and feeding characteristics of the medical food. The fact that these people are tube-fed should not work to their nutritional detriment. Nondigestible oligosaccharides, which are highly soluble and rapidly fermentable, were identified as ideal sources of dietary fiber for use in medical foods.

An oligosaccharide is a carbohydrate consisting of a small number, from 2 to 10, of monosaccharides. Oligosaccharides can be divided into two broad categories: digestible and nondigestible. Many types of nondigestible oligosaccharides are produced commercially from various sources of food materials (Table I). Most of these oligosaccharides are reducing sugars, and thus, they are susceptible to the formation of Maillard products during liquid product manufacturing. This non-enzymatic browning reaction forms linkages that are not hydrolyzed during digestion resulting in the loss of amino acid availability (*3*). Any oligosaccharide that contains reducing sugars would be susceptible to the formation of Maillard products with free α-NH_2 groups and especially the ε-NH_2 group of lysine.

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Table I. Nondigestible Oligosaccharides

Oligosaccharide	*Source/origin*	*Reducing sugar*
Short chain fructooligosaccharides	sucrose	no
Hydrolyzed inulin[1]	inulin	yes
Xylooligosaccharides	xylan	yes
Soybean oligosaccharides	soybeans	no
Galactooligosaccharides[2]	lactose	yes
Lactulose	lactose	yes

[1] Also referred to as oligofructose (*4-5*).

[2] Also referred to as transgalactosylated oligosaccharides (*6*).

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Short chain fructooligosaccharides (scFOS) occur naturally and have been isolated from such foodstuffs as onions, wheat, barley, bananas, tomatoes, garlic, and artichokes (*7-10*). The isolation and development of scFOS was first reported in the Japanese literature in 1983 (*11 cited from 12*). Although scFOS can be extracted from a variety of plants, they can also be produced by adding *Aspergillus niger* fructosyltransferase (β-fructosyltransferase) to sucrose (*13*). Short chain FOS (e.g., Neosugar, Nutraflora™, Meioligo®, Actilight®) consist of the following oligosaccharides: 1-kestose, nystose and 1^F-β-fructofuranosyl nystose. These oligosaccharides consist of two to four fructofuranosyl units linked by β-(2→1) glycosidic bonds and carry a single α-D-glucopyranosyl unit at the non-reducing end of the chain, linked (1↔2) as in sucrose. Short chain FOS are non-reducing sugars and will not undergo the Maillard reaction. The Ross Products Division of Abbott Laboratories incorporates scFOS into several medical foods. The application of scFOS to medical foods as a fermentable dietary fiber is discussed below.

Fermentation of Fructooligosaccharides

The physiological effects of scFOS are directly related to the fact that they are not digested in the upper gastrointestinal tract but remain intact as they enter the large bowel where they are fermented by the indigenous microflora. A number of studies have shown that the digestive enzymes of vertebrates do not hydrolyze scFOS. Hydrolysis of scFOS could not be demonstrated during *in vitro* incubations with either human jejunal homogenates (*14*) or human salivary enzymes (*11*). Furthermore, scFOS were shown to be nondigestible in rats (*15*) and humans (*11,16*). While mammalian enzymes are unable to degrade scFOS, there exists within the gastrointestinal tract a large and diverse microflora population capable of utilizing scFOS as an energy source.

Fermentation, the process by which anaerobic organisms break down dietary and other substrates to obtain energy for growth and the maintenance of cellular function, is an important component of large bowel activity (*17*). More than 70% of the energy from carbohydrate fermentation is conserved as short chain fatty acids (SCFA) and other fermentation products (methane, carbon dioxide, and hydrogen) (*18*). The SCFA serve as a source of energy for the host. Acetate, the most abundant SCFA, is primarily used as a fuel for host tissues. It is the only SCFA found at appreciable levels in the peripheral blood where it may be oxidized in muscle and adipose tissue. Propionate may be oxidized by the colonocytes as a source of energy, but it is believed to be primarily used by the liver as a substrate for gluconeogenesis (*19*). Butyrate is preferentially oxidized by colonocytes as a source of energy. Roediger (*20*) found that more than 70% of the oxygen consumed by colonocytes from the ascending and descending colon could be attributed to butyrate oxidation in isolated human colonocytes.

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Human colonic bacteria rapidly ferment scFOS. Wolf *et al.* (*21*) compared the fermentability of several nondigestible oligosaccharides by human fecal inoculum *in vitro* including: scFOS, xylooligosaccharides (XOS), and hydrolyzed inulin. Short chain FOS and hydrolyzed inulin were completely fermented by 6 hours, and XOS was completely fermented by 12 hours (Figure 1). A similar study evaluating the fermentability of soy fiber, gum arabic, scFOS and lactulose was conducted by Garleb *et al.* (*22*). Lactulose and scFOS were fermented much more rapidly ($P < 0.01$) than either gum arabic or soy fiber. Furthermore, the fermentation of scFOS was essentially complete by 6 hours and by 12 hours for lactulose.

Physiological Effects

As mentioned previously, the structural characteristics (i.e., nondigestible in the upper gastrointestinal tract, non-reducing, and highly soluble) of scFOS make for an ideal fermentable fiber source in medical foods. Potential physiological benefits of scFOS for patients include positive effects on bowel

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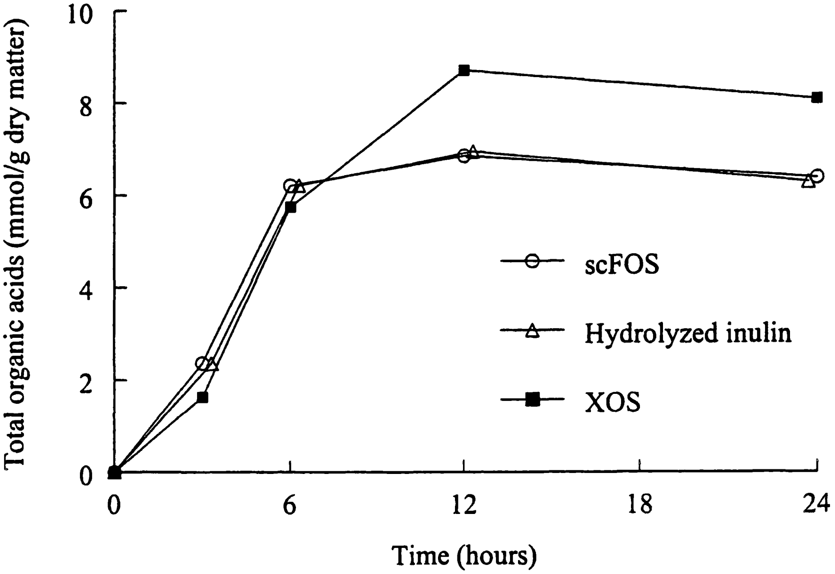

Figure 1. Total organic acid (acetate, propionate, butyrate and lactate) production from in vitro fermentation of nondigestible oligosaccharides with human fecal inoculum (n = 3). Data are from reference 21.

function, large bowel integrity, colonization resistance, nitrogen excretion and calcium absorption.

Bowel Function

Of all the physiological benefits of dietary fiber, its effect on bowel habits is the most noted. The effect of dietary fiber can best be described as a "normalization" of bowel function because it has been used for the treatment and prevention of both constipation (*23*) and diarrhea (*24*). Human studies confirm that the consumption of scFOS can affect stool consistency. Hata and Nakajima (*25*) administered varying single doses of scFOS to 80 healthy adults and determined that the dosage resulting in 50% of the subjects experiencing diarrhea (a watery stool during the first defecation following ingestion of scFOS) was approximately 0.8 g of scFOS/kg body weight. Subsequent studies conducted by Briet *et al.* (*26*) in which healthy human adults received acute or chronic dosing with scFOS generated similar results. Tokunaga *et al.* (*27*) showed that healthy volunteers had softer stools and increased frequency of bowel movements when they consumed scFOS for two weeks (up to 5 g/day) than during baseline or after a week washout period. The administration of scFOS has been reported to relieve moderate cases of constipation (*11,28*); however, severe constipation was not alleviated (*28*).

Short chain FOS, through fermentation and production of SCFA, may play a positive role in the alleviation of diarrhea. Short chain fatty acids can improve bowel function by facilitating water absorption. The absorption of 100 mmol SCFA is associated with the absorption of 360 ml water (*29*). Ramakrishna and Mathan (*30*) found that fecal output of SCFA in patients with acute diarrhea was low on the first day of illness, but increased over the next five days as the patients' condition improved. Furthermore, they demonstrated that luminal SCFA could restore net water and sodium reabsorption in the rectum of patients with acute diarrhea. *In vivo* perfusion studies in healthy subjects have shown secretion of salt and water in the ascending colon in response to enteral feeding (*31-32*). Bowling *et al.* (*33*) investigated the effect of SCFA on colonic fluid secretion induced by enteral feeding. These researchers found that SCFA infusion into the cecum of healthy subjects reversed the fluid secretion seen in the ascending colon during enteral feeding and theorized that these findings could have implications for the management of diarrhea related to enteral feedings.

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Large Bowel Integrity

Short chain FOS, through the production of SCFA, may be useful in maintaining large bowel integrity or serving as adjunctive therapy for the treatment of inflammatory bowel disease. The feeding of dietary fiber has been associated with the stimulation of colonic cell proliferation. The stimulatory effect has been attributed to the SCFA produced during fiber fermentation. Aghdassi *et al.* (*34*) found that reducing colonic fermentation resulted in reduced intestinal adaptation and nutritional recovery in rats with massive small bowel resection. Moreover, others have observed stimulation of small bowel mucosal proliferation with SCFA supplementation (*35-36*). Younes *et al.* (*37*) fed rats a fiber-free diet or diets containing 7.5% fiber as oat fiber, gum arabic, scFOS, or XOS. Cecal wall weight was greater ($P < 0.05$) for the fermentable substrates (i.e., gum arabic, scFOS and XOS) compared with the poorly fermented oat fiber and the fiber-free control. Similarly, Campbell *et al.* (*38*) fed rats a fiber-free diet or 5% cellulose diets with or without 6% scFOS, hydrolyzed inulin, or XOS. Nondigestible oligosaccharide supplementation increased ($P < 0.05$) cecum wall weight compared with the fiber-free and cellulose-containing control diets. Howard *et al.* (*39*) fed supplemental (3 g/L) scFOS in a liquid diet to neonatal pigs and observed increased ($P < 0.01$) cell proliferation in the proximal and distal colonic epithelial mucosa. Bourquin *et al.* (*40*) evaluated cecal and colonic development of neonatal pigs ($n = 10$) fed sows milk, milk replacer, or milk replacer plus 3 g of scFOS/L for 14 days. Cecal tissue weight was greatest ($P < 0.05$) for pigs consuming milk replacer + scFOS compared with pigs consuming either milk replacer alone or sows milk (Figure 2). Colonic tissue weight was greatest for pigs consuming milk replacer plus scFOS, least for pigs nursing the sow, and intermediate for pigs consuming milk replacer alone. From these animal experiments, it can be concluded that fermentable substrates may play a key role in the maintenance of large bowel integrity and function.

Increasing interest has been generated in the use of enemas/irrigation solutions containing buffered, physiologic levels of SCFA for the treatment of diversion colitis and ulcerative colitis. Diversion colitis is an inflammatory process arising in segments of the colorectum at various intervals after surgical diversion of the fecal stream. The endoscopic appearance is similar to those of active Crohn's disease and ulcerative colitis (*41*). The cause of this condition is not known, but one mechanism has been postulated: a nutritional deficiency of the colonic epithelium, specifically due to the absence of SCFA normally present in colonic contents (*42-43*). Harig *et al.* (*44*) tested this hypothesis by assessing whether SCFA irrigation could ameliorate inflammation in four patients with diversion colitis. After two to three weeks of therapy, macroscopic and histological resolution of inflammation was evident.

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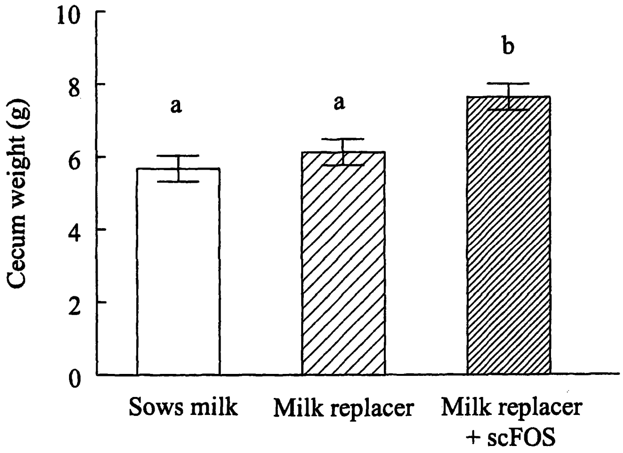

Figure 2. Cecum weight of neonatal pigs fed sows milk, milk replacer, or milk replacer + 3 g of scFOS/L. All values are mean ± pooled SEM, n = 10. Bars with unlike superscript letters differ ($P < 0.05$). Data are from reference 40.

Impaired utilization of SCFA, which has also been implicated in ulcerative colitis, suggests that diminished intracellular energy production may be important in the inflammatory process (*45*). It has been demonstrated that fecal water from patients with ulcerative colitis contains reduced concentrations of SCFA as well as markedly increased lactate concentrations and decreased pH (*46-47*). In a study by Breuer *et al.* (*48*), the effect of large bowel irrigation with SCFA in patients with ulcerative colitis was studied. It was found that 9 out of 10 patients completing the study were judged to be at least much improved and showed a significant change in mean disease activity index score and mucosal histology score. Senagore *et al.* (*49*) confirmed these results by demonstrating an 80 percent response rate in patients with idiopathic proctosigmoiditis. This study indicates that administering a solution of SCFA for six weeks was equally efficacious to corticosteroid or 5-aminosalicylate for the treatment of proctosigmoiditis at a significant cost savings. Scheppach *et al.* (*50*) investigated the use of butyrate enemas alone rather than the SCFA mixture to treat ten patients with distal ulcerative colitis in a placebo-controlled, single-blind, randomized trial. The authors concluded that butyrate markedly improved disease activity index and histological parameters. These data support the hypothesis that the effect of a SCFA mixture on the inflamed mucosa in ulcerative colitis is largely attributable to its butyrate moiety.

It is unlikely that SCFA added directly to an enteral product would reach the large bowel. Medical foods can take advantage of the positive effect of SCFA by providing fermentable fiber. For example, Rolandelli *et al.* (*51*) demonstrated a benefit of a fermentable fiber (pectin) in the treatment of experimental colitis. Also, Grisham *et al.* (*52*) evaluated the effect of enteral diets containing fish oil or the fermentable substrates, scFOS or XOS, on chronic colitis induced with peptidoglycan polysaccharide in rats. Histological and biochemical markers of inflammation were improved. Seidner *et al.* (*53*) assessed the efficacy of a novel medical food supplemented with fish oil, scFOS, gum arabic, and antioxidants on reducing corticosteroid use in adults with mild to moderate ulcerative colitis. Patients given this formula had a significantly greater rate of reduction in the daily dose of prednisone over 6 months as compared with controls receiving a sucrose-based placebo.

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Prebiotics and Colonization Resistance

Short chain FOS are a prebiotic: a nondigestible food ingredient that improves host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (*54*). *In vitro* experiments indicate that scFOS are utilized by several beneficial species of gastrointestinal bacteria, but not utilized by potentially pathogenic species. Using pure cultures, Hidaka *et al.* (*11*) showed that bacteroides and bifidobacteria grew to high optical densities and produced large amounts of organic acids when scFOS were provided as an energy source. However, potential pathogens such as *Escherichia coli* and *Clostridium perfringens* did not.

Enrichment for bifidobacteria in human feces has also been demonstrated *in vivo*. When scFOS were fed to elderly Japanese patients, bifidobacteria numbers in feces increased approximately 10-fold (*55*). In healthy adult volunteers, Williams *et al.* (*56*) reported an increase in fecal bifidobacteria numbers with the consumption of 4 g of scFOS/day. A study conducted by Garleb *et al.* (*57*) further documented the bifidogenic capacity of scFOS. In this study, healthy subjects were fed a low residue polymeric formula (LRPF), LRPF + 5 g of scFOS/L (~15 g of scFOS/day), or LRPF + 10 g of scFOS/L (~31 g of scFOS/day) for two weeks. The scFOS had a significant impact on fecal bifidobacteria levels. On day 14, a greater number ($P < 0.001$) of bifidobacteria were detected in the feces of subjects consuming formulas containing scFOS compared with those subjects consuming the LRPF without scFOS.

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Colonization Resistance

Indigenous bacteria confer colonization resistance to the host. In the gastrointestinal tract, this phenomenon refers to the ability of the indigenous microflora to prevent the colonization, overgrowth and/or translocation of potentially pathogenic microorganisms. This concept was first described by Van der Waaij *et al.* (*58*) who showed that indigenous anaerobic organisms prevented colonization by opportunistic pathogens.

An excellent example of colonization resistance is the relationship between indigenous bacterial microflora and *Clostridium difficile*. *C. difficile*, a spore forming obligate anaerobe, is the leading known cause of nosocomial diarrheal infections (*59-60*). When established in the colon, pathogenic strains of *C. difficile* produce exotoxins (toxin A and toxin B) that are the cause of diarrhea and colitis (*61-62*). Infections due to *C. difficile* are responsible for all cases of pseudomembranous colitis (PMC) and for up to 20% of cases of antibiotic-associated diarrhea without colitis (*63-64*).

Use of almost any antibiotic can cause *C. difficile* infection, but broad-spectrum antibiotics with activity against enteric bacteria are the most frequent agents. This causative factor suggests that *C. difficile* infections are associated with the disruption of the normal bowel microbiota. Wilson *et al.* (*65*) noted that *C. difficile* could not colonize hamsters in the presence of an undisturbed colonic microflora, yet *C. difficile* rapidly attained a large population size when introduced into antibiotic-treated animals. Likewise, *C. difficile* was able to achieve a population of over 10^8 CFU per cecum when inoculated into

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gnotobiotic mice, but colonization was suppressed to undetectable levels by intestinal flora of conventionally colonized hamsters and mice (*66*).

Standard antibiotic therapy (i.e., vancomycin or metronidazole) is effective in 80% of patients with *C. difficile*-associated disease, but the remaining 20% experience further episodes of diarrhea or colitis during the permissive period after the antibiotic has been discontinued (*67-69*). Once patients have had one recurrence, they may experience repeated episodes of the disease over several years (*70-71*).

Given the causative role of antibiotics in the onset of *C. difficile* infection, alternative therapies might offer more effective strategies for the prevention or treatment of *C. difficile* infection. Promising results have been obtained by restoring normal gut flora. Schwan *et al.* (*72-73*) effectively treated recurrent *C. difficile* infection by giving enemas with fecal contents from healthy adult humans. While direct inoculation with gastrointestinal microflora appears effective, its acceptability in a clinical setting is questionable. A better approach may be to use fermentable fibers such as scFOS to restore the gastrointestinal microflora and environment.

In an *in vitro* model, May *et al.* (*74*) showed that fermentable fiber (resulting in increased SCFA concentrations and decreased pH) effectively inhibited the growth of *C. difficile* and toxin A production. In addition, May *et al.* (*75*) found that dietary supplementation with oligosaccharides (scFOS or XOS) suppressed the growth of *C. difficile,* protected the cecal epithelial tissue, and reduced the incidence of diarrhea in *C. difficile*-challenged mice. Gaskins *et al.* (*76*) demonstrated that the administration of scFOS to cefoxitin treated mice inoculated with *C. difficile* produced toxin A titer in feces significantly lower ($P < 0.05$) than animals not receiving scFOS and similar to mice not compromised with antibiotics. Wolf *et al.* (*77*) demonstrated that dietary supplementation with scFOS increased survival time in a hamster model of *C. difficile*-colitis (Figure 3). It is interesting to note that the benefit due to the supplementation of scFOS in this study was above any effects of fiber contained in the basal diet. In consideration of the research conducted in this area, the addition of scFOS to a medical food may be beneficial for patients at risk of *C. difficile* infection in long-term care institutions and hospital wards.

Nitrogen Excretion and Calcium Absorption

The addition of fiber to a diet can potentially alter total body nitrogen metabolism by enhancing bacterial metabolism and thereby increasing the incorporation of nitrogen into fecal bacteria. This in turn increases fecal nitrogen excretion and subsequently reduces urinary nitrogen excretion (*78-79*).

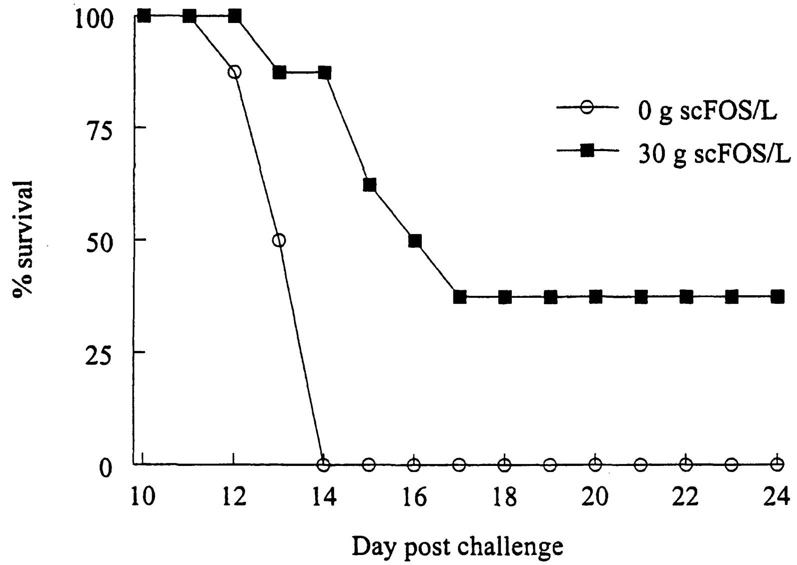

Figure 3. Survival curves for ciprofloxacin treated hamsters (n=8) receiving either 0 or 30 g of scFOS/L of drinking water. (Reproduced with permission from reference 77. Copyright 1997 Japan Bifidus Foundation.).

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For example, lactulose has been reported to depress ammonia absorption from the large bowel (*80*) and to enhance fecal nitrogen excretion (*81*). In addition, inulin has been shown to enhance urea capture by the rat cecum (*82*) and promote fecal nitrogen excretion, particularly when the level of protein in the diet is moderate (*83*). Younes *et al.* (*37*) demonstrated in rats that fermentable fibers such as gum arabic, XOS, and scFOS could increase fecal nitrogen excretion at the expense of urinary nitrogen excretion. Such data indicate a potential benefit for nondigestible oligosaccharide therapy in patients with renal insufficiency or chronic renal disease.

Considerable efforts are underway to encourage all people, particularly women, to increase their calcium consumption for improved bone health. Ingredients that improve mineral absorption also could be beneficial. Fermentable fibers such as inulin (*83*), hydrolyzed guar gum (*84*) and scFOS (*85-87*) have been shown to enhance calcium absorption. This enhanced absorption occurs in the large bowel (*85-86,88*) through a variety of mechanisms that may involve SCFA (*89*), pH (*90*), and calbindin-D9K (*88*).

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Conclusions

Short chain fructooligosaccharides represent an ideal source of fermentable fiber for use in medical foods. Short chain fructooligosaccharides are highly soluble fibers that will not increase the viscosity of liquids and compromise the tube feeding characteristics of a medical food. *In vitro*, animal, and in several instances, human research has been conducted to identify and support the potential physiological benefits of short chain fructooligosaccharides.

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Chapter 11

Fructooligosaccharides and Other Fructans: Structures and Occurrence, Production, Regulatory Aspects, Food Applications, and Nutritional Health Significance

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The non-digestible fructans, particularly inulin and their subset the fructooligosaccharides (FOS), possess a number of highly desirable attributes, such as no carigenicity, safe for diabetics, low calories, selective source of dietary fiber, and strong bifidus-stimulation. They further add to the functionality in food products by adding texture, mouthfeel, taste improvement, and can help replace sugar and/or fat. These healthy ingredients are widely distributed in nature, being only second to starch in their occurrence, and can positively contribute to the health of individuals in several physiological areas. These molecules have been shown to be excellent prebiotic fiber sources, selectively enhancing the growth of healthy gut microflora, while suppressing the growth of pathogenic bacteria, to demonstrate several key health implications.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

A rapidly aging population having a desire to be healthy into senior years has helped fuel a growing demand for healthier and calorie-controlled foods, made from highly functional and nutritional non-digestible oligosaccharides (NDO) created since the 1980s. Considering all of these new functional products introduced so far, the fructans, specifically inulin and fructo-oligosaccharides (FOS), have attracted special attention. The inulin-type fructans, perhaps more than any other fiber source, have extensive clinical research elucidating their physiological and nutritional characteristics, which spans more than a century. Further, their physicochemical properties as they relate to food product development are widely known. These molecules when used to produce food products can add texture and mouthfeel, improve taste, can help replace sugar and/or fat and act as filler/binders in tablets. In addition, their physiological health-promoting properties have demonstrated significant potential health implications. Most promising areas of health promotion are those related to gastrointestinal function, enhanced calcium absorption, with potential osteoporosis implications, and enhanced immune functions and anti-tumor activity. These dietary components also have been shown to provide systemic influence in modulating lipid and carbohydrate metabolism for better cardiovascular and diabetic health implications.

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Definition, Origin, Biosynthesis, Intakes and Legal Status

Fructans are widely distributed as a carbohydrate reserve among a variety of higher plants, involving 9 mono- and 14 dicotyledonous families (*1*). After starch, they are the most abundant storage carbohydrates in the plant kingdom (*2*). Included within these families are several food plants containing inulin-type fructans having extensive documented historical human use, such as wheat, onion, leek, bananas, garlic, raisins, yacon, artichokes and chicory (*3*). Fructans also are found occurring naturally in many micro-organisms, primarily of the levan-type. These micro-organisms include notable gram positive bacteria, the *Bacillus, Streptococcus, Rothia, Lactobacillus, Arthrobacter, Actinomyces and Azotobacter,* while notable gram negative bacteria are the *Erwinia, Zymomonas, Pseudomonas,* and *Gluconobacter* (*4*). In addition, the fungi *Aspergillus, Auerobasidium,* and *Fusarium,* the mold *Penicillium* and the yeast *Saccharomyces* also produce levan-type fructans.

Historically, daily inulin intake from the consumption of edible plants was estimated to be approximately 25 to 32 grams. However, today the average daily intake of inulin and its hydrolysis products in Western Europe is estimated between 2-12 g/person/day (4). The US inulin consumption was estimated at 2-8 g/person/day, based on data from the US Nationwide Food Consumption Survey

1987-88 (*5*). More recently a study by Moshfegh and others of the USDA (*6*) showed that American diets provide about 2.6 g/person/day and 2.5 g/person/day of inulin and its partial hydrolyzate, oligofructrose, respectively, mostly coming from the consumption of wheat and onions in the diet.

As inulin-type fructans are classified as food ingredients worldwide (non-additive or E-number status), as FOSHU in Japan and GRAS in the USA, they are incorporated *ad libitum* in all food categories. Due to their unique physicochemical and physiological properties these ingredients are further categorized as part of the dietary fiber complex and are being used for improving food quality and providing highly nutritional foods that can influence the health of world populations (see following sections on food applications and nutritional health implications). Moreover, their content may be specifically measured analytically in food and food products using approved AOAC Fructan Methods 997.08 and 999.03.

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Fructans are generally defined as being a polymer of fructose having more than 10 fructose units. In plants, up to 200 fructose units can be linked in a single fructan molecule. By contrast, bacterial fructans are much larger, with as many as 10,000 – 3M residues. But, unlike glucose polymers, fructose molecules, have no clear distinction between oligomers and polymers and some fructans of the same type have reported lengths distributed between 6 and 260 units. Due to this, the oligomeric molecules, beginning with dimers of fructose, are considered to be fructans, regardless if the fructose ring has a furanose form (*7*). It should be noted that in its broadest definition fructose can also be termed a fructan.

As more commerical products become available, it is important to have an understanding of the different terms used to describe commercially-significant fructose-containing polymers. Fructooligosaccharides (FOS) naturally occurring in many plants, such as the brans of triticale, wheat, and rye, and as produced in mass using food grade enzymes on sucrose, are typically of the GF_n – type (neosugar) molecules, being mainly composed of 1-kestose (GF_2), nystose (GF_3), and 1^F-fructofuranosyl nystose (GF_4), in which the fructosyl units (F) are bound at the β-2,1 position of sucrose (GF). The n typically is between 2 to 4 fructose units. Fructooligosaccharides or oligofructose (*8*) derived from partial enzymatic hydrolysis of native chicory inulin are a mixture of homopolymers of fructose (F_m-type molecules) and GF_n –type molecules, in which the fructosyl moieties (F_m and F_n) are bound by a β-(2→1)-linkage. The ratio of GF_n to F_m is approximately 60:40, where the n and m values typically range between 2 to 8 fructose units. The average degree of polymerization (DP) for the product being about 4-5 monomeric units. The predominant native commercial inulin product originates from chicory root (*Cichorium intybus*) and is a GF_n – type molecule, in which the fructosyl units (F) are linked via a β-(2→1)-bond. The molecule has a terminal glucose unit with n ranging between 2 to 60 fructose units having a modal DP of approximately 10 monomeric units. Other fructan products are also commerically available, but currently are not in mass worldwide use.

In nature, the various fructans are distinguished on the basis of the glycosidic linkages present between the fructose residues. These types are broadly classified into 3 groups, the inulins, the levans (or phleins or phleans), and mixtures or highly branched chain fructans referred to as the graminan type. The first group, the inulin type fructans, are characterized as being predominately linear molecules, made up of fructosyl units linked via a β-(2→1)-bond. The molecule is typically terminated by a glucosyl unit bound to one of the fructose moieties via an α1-β2 type linkage, as in sucrose. The inulin-type fructan may also contain a small degree of branching through a β-(2→6)-linkage in some plants (*9*). All fructans found in the dicotyledons, as well as some monocotyledons are of this type. Figure 1 shows 1-kestose, the simplest form of this type. The chain length or degree of polymerization (DP) can vary widely depending on the plant source and its level of maturity.

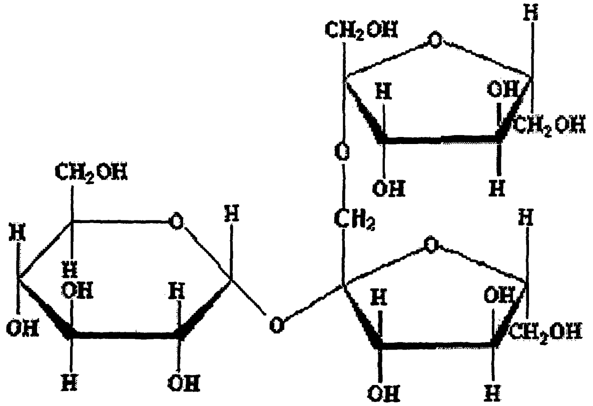

Figure 1. 1-kestose

The second group are the levans, also linear fructans. However, these fructans are predominately made up of fructose moieties linked via a β-(2→6)-bond (*10*). A terminal glucose unit may also exist in this type, but it is more difficult to establish if this exists in all levans due to the high molecular weight, leaving glucose as a very small fraction of the total material (*7*). The levan-type fructan may also contain a small degree of branching through a β-(2→1)-linkage. This type of fructan is found in many of the monocotyledons and in

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almost all bacterial fructans. Figure 2 shows 6-kestose, the simplest form of the levan type.

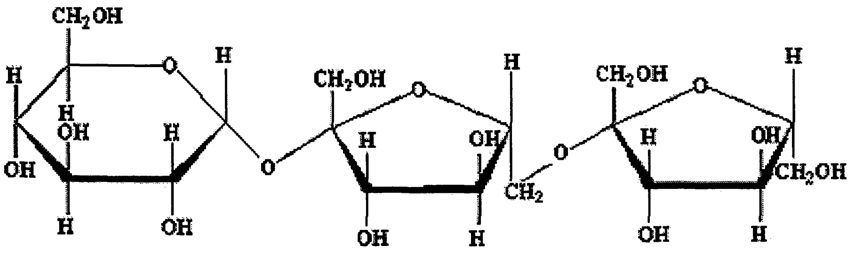

Figure 2. 6-kestose.

The third group are the fructans of mixed type, also referred to as the graminan or "grass" type (*2,11*). This group is distinguished as having significant quantities of both β-(2→1) and β-(2→6)-linked fructose units, and thus contain significant branching. As the name refers, this type is common to the grasses. Figure 3 shows 6,6,1-kestopentaose, the simplest form of the graminan fructan type.

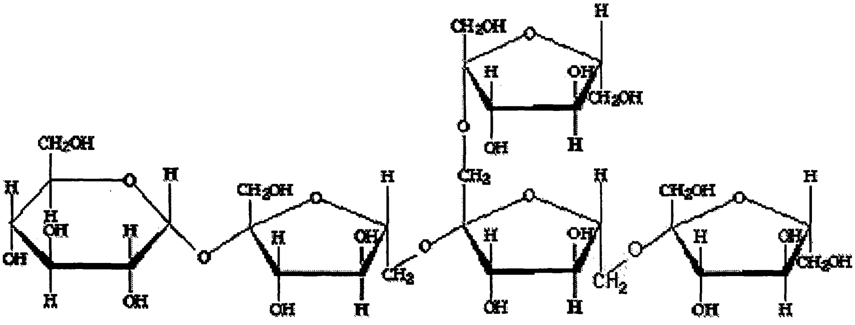

Figure 3. 6,6,1-kestopentaose.

Fructans are naturally produced by 15% of the flowering plant species and many bacterial genera. Plant species typically storing fructans, from an evolutionary standpoint, tend to be represented by many of the most advanced families and are often located in regions with seasonal drought or cold. It has

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been proposed that the function of fructan in plants, being highly soluble in water and thus more mobile within the plant (osmotically active), could be as an osmoprotectant against drought and cold. Unlike starch, which is stored in the plastids, fructans are normally stored in the plant vaculoes (*12*). By changing the degree of polymerization of the molecule in the plant's vacuole, the plant can readily change the osmotic potential of its cells without altering the total amount of carbohydrate.

In a review of the fructan synthesis in plants *in vivo,* Pollock and others (*13*) noted that the natural biosynthesis of fructans in plants, primarily of the inulin type, takes place using two vacuolar enzymes, 1-SST (sucrose-sucrose fructosyltransferase) and 1-FFT (fructan-fructan fructosyl transferase). 1-SST catalyzes the reaction in Equation 1 that produces trisaccharides (1-kestose, 6-kestose, and neo-kestose) and a glucose molecule from two sucrose molecules (*14,15*). Following trisaccharide production, the polymeric chain is lengthened by the action of 1-FFT, catalyzing the transfer of a unit of fructose from one donor trisaccharide from the 1-SST process to another receptor, releasing sucrose, and thus elongating the chain (equation 2). 1-SST and 1-FFT thus have some overlapping activities. During this transfer sucrose molecules only act as acceptors and cannot function as donars. The overall reaction mechanism is expressed as:

$$\mathrm{GF} + \mathrm{GF} \Rightarrow \mathrm{GF{-\!\!-}F} + \mathrm{G} \qquad \text{by SST} \quad (1)$$

$$\mathrm{G(F)_n} + \mathrm{G(F)_m} \Rightarrow \mathrm{G(F)_{n-1}} + \mathrm{G(F)_{m+1}} \qquad \text{by FFT} \quad (2)$$

where GF is a sucrosyl group and *n* is the number of extrasucrosyl fructose residues. The *m* represents the number of fructosyl moieties in the homopolymers of fructose.

The FFT enzymes are probably also responsible for structural diversity of fructans in nature, because FFTs are responsible for fructans having more than two fructose moieties. Concerted action of a variety of FFT-activities leads to structurally different fructans. By example, plants producing inulins of only the neokestose type use the concerted action of 1-SST, 6-glucose-FFT and 1-FFT, while those plants producing predominately levan-type fructans use 6-sucrose-fructosyltransferase (6-SFT). The plants producing mixed-type levans typically use 1-SST, followed by 6-SFT and 1-FFT and 6-SFT. Two other enzymes involved in fructan metabolism are also FH (fructanhydrolases) and invertases. FH, as a β-fructofuranosidase, can cleave fructose units from fructans, releasing sucrose (*16*). Invertase, an active vacuolar enzyme, cleaves sucrose to glucose

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and fructose, and can also cleave fructose molecules from smaller fructans. As degree of polymerization increases this fructan dehydrolysing activity decreases.

In contradication to the plant genes for enzymes associated with fructan metabolism, bacterial genes are cloned and their sequence is defined. By contrast to plants, only one enzyme is responsible for the trisaccharide synthesis and elongation of fructans in bacteria. Various levansucrases (LS) and fructosyltransferases (FTF) have been studied. LS, which synthesizes levans, was isolated from *Bacillus subtilis* (*17*), and FTF, which synthesizes inulin type fructans, was isolated from *Streptococcus mutans* (*18*). The LS and FTF catalyzed reactions, using sucrose are shown in equation 3. LS also has invertase activity, which catalyzes the hydrolysis of sucrose to its monomeric components, glucose and fructose, equation 4.

$$GF + G(F)_n \Rightarrow G + G(F)_{n+1} \qquad (3)$$

sucrose + levan (n) ⇒ glucose + levan (n+1)

$$GF + H_20 \Rightarrow G + F \qquad (4)$$

Sucrose + water ⇒ glucose + fructose

Production

As mentioned previously, the majority of the fructans produced commerically and being used for food and animal feed development, are of the inulin type. The primary industrial source of pure inulin and its partial hydrolyzate, oligofructose, is the chicory root (*Cichorium intybus* L.), while FOS of the GF_2, GF_3, and GF_4 type are produced by transfructosylation of sucrose using β-fructosyltransferease enzyme derived from microorganisms or plants.

Native inulin and oligofructose originating from the chicory root, a plant similar to the sugar beet, is produced on an industrial scale using steps rather similar to that of sugar extracted from sugar beets. The roots are usually harvested, sliced and washed. The native inulin is then hot water extracted via diffusion, then purified, and finally spray dried. The resulting product has a modal degree of polymerization (DP) of 10-14 and a distribution of molecules having chain lengths ranging from 2-60 fructosyl residues (Figure 4). Oligofructose is derived from chicory root in much the same manner, but, as noted earlier, is a partial hydrolyzate of the native inulin, where the chicory raw juice is subjected to an enzymatic hydrolysis step after extraction using endoinulinase (Figure 4). The resulting oligofructose has chain lengths ranging from 2 to 8, with an average DP of 4.

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Fructooligosaccharide production by transfructosylation of sucrose using β-fructosyltransferase enzyme can be divided into two classes: the first is the batch system using soluble enzyme and the second is the continuous process using immobilized enzyme or whole cells. Meiji Seika Co. (Japan), who initially produced FOS, have utilized a continuous process involving the immobilization cells of *Aspergillus niger* entrapped in calcium alginate gel. In 1990, Cheil Foods & Chemicals Co. (South Korea) developed and has utilized a continuous process, but utilized immobilized cells of the fungus *Aureobasidium pullulans* entrapped in calcium alginate gel. The batch and continuous process of commercial FOS production are shown in Figure 5. Several adsorbents and ion-exchange resins have been studied for fructosyltransferase immobilization (*19-22*) in combination with alginate gel (*23-26*). Fructans formed by these processes contain 2-4 fructose moieties linked to a terminal glucose. The glucose and fructose molecules formed as by-products of the process, as well as any unreacted sucrose, may be removed by the use of chromatography employing columns such as carbon Celite (*27*). Typical refined FOS products contain about 5% residual sugars.

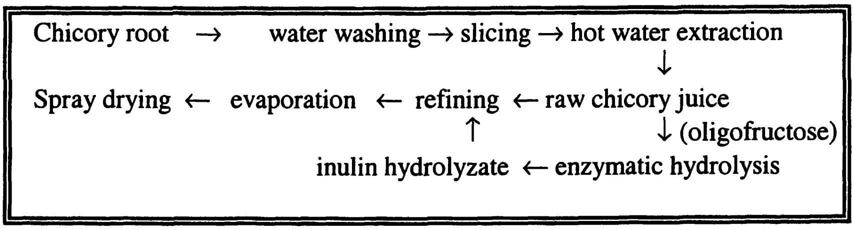
Chicory root → water washing → slicing → hot water extraction
↓
Spray drying ← evaporation ← refining ← raw chicory juice
↑
↓ (oligofructose)
inulin hydrolyzate ← enzymatic hydrolysis

Figure 4. Inulin and oligofructose manufacturing process.

Functional Properties

The diversity in molecular configuration and chain length between the various fructans help define their distinctly different functional and physiological attributes. The bacterial levans, typically being relatively long chain molecules have not seen any significant application in the food industry. Levan from *Aerobacter levanicum*, has been effectively used as a non-toxic, non-antigenic blood volume expander when reduced to suitable molecular size. In addition, levan from *Bacillus polymyxa*, due to its relatively long chain length, has been proposed as a food thickener, sweetness potentiator, flavor encapsulator or a source for fructose syrup production (*28*).

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Inulin-type fructans have seen significant use in mainstream food product development, food-based dietary supplements, and substantial interest in animal feed formulations. Due to the long chain length of inulin, relative to FOS, inulin is less soluble than FOS or oligofructose, and has the ability to form microparticulate crystalline gels when hydrated and sheared in water or milk systems. Being submicron in size, these microcrystals are not discernible as individual particles in the mouth and provide a creamy texture and a fat-like mouthfeel. Due to this property, chicory inulin has been successfully used in synergy with other hydrocolloids to replace fat with improved rheological properties in many food systems.

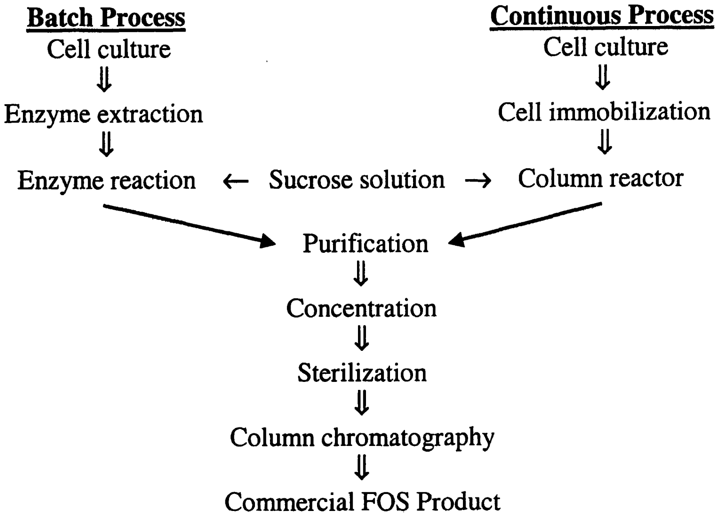
Batch Process
Cell culture
Enzyme extraction
Enzyme reaction
Sucrose solution
Continuous Process
Cell culture
Cell immobilization
Column reactor
Purification
Concentration
Sterilization
Column chromatography
Commercial FOS Product

Figure 5. Production outline of FOS via transfructosylation of sucrose.

Shorter chain fructooligosaccharides possess functional qualities similar to sugar or glucose syrups. FOS contributes bulk to dairy products and humectancy to soft baked products, provides colligative properties to depress freezing point in frozen desserts. Native inulin and FOS both can be used as effective binders and provide low calorie fiber sources in beverages, health bars and confection applications, with used levels being dependent on application. Inulin and FOS are often used by themselves or in combination with other low caloric non-sugar bulking agents, such as polyols and other low viscosity fiber sources, and/or high intensity sweetners to replace sugar. The use of inulin and FOS has been shown to provide a desirable well-rounded sweetness profile and mask the aftertaste of

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several high intensity sweeteners, such as aspartame, acesulfame potassium, saccharin, and cyclamates (*29*).

Inulin-type fructans are used worldwide to add selective fiber to various food products. Unlike many other fibers, these non-digestible carbohydrates are unique by not contributing to objectionable flavor profiles or significantly increasing the viscosity of a food system. Their properties allow high fiber-containing food products to be developed that have lower fat and/or sugar, are lower in calories and more healthy, but still look and taste like their more standard food counterparts.

The nutritional properties of inulin and FOS are similar; so the physicochemical properties of each and the desired attributes of the finished product define the choice or blend of their use.

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Nutritional Properties

Non-digestibility and Physiological Significance.

The 1,2 and 2,6-β-linkages making up inulin, FOS, and levans are resistant to mammalian digestive enzymes, such as the disaccharidases (sucrase, maltase, isomaltase or lactase) of intestinal mucosa and α-amylase of pancreatic homogenates (*30,31*). Many studies have shown that inulin-type fructans pass through the human digestive system without being metabolized (*32-37*). Consequently, these dietary components reach the colon virtually unaltered. Fructans of the inulin-type are categorized with other fermentable dietary fibers in the dietary fiber complex since they are not hydrolyzed by the human digestive system, but are completely fermented selectively by health-promoting colonic microflora, bifidobacteria and lactobacilli. Health benefits ascribed to the bifidobacteria include: competitively inhibiting the growth of harmful microorganisms, such as *Salmonella, Shigella, Clostridia, Staphylococcus aureus, Candida albicans, Campylobacter jejuni, E. coli, Veillonella and Klebsiella;* stimulating components of the immune system, lowering blood cholesterol and triglycerides; reducing liver toxins, reducing food intolerances and allergies; aiding the absorption of certain minerals, like calcium; and synthesizing B-vitamins. The dietary fiber influence of inulin-type fructans also have positive intestinal effects and positive benefits on lipid and carbohydrate metabolism. Research has shown that the daily consumption of a few grams of inulin-type fructans per day in the diet will cause dramatic shifts in the distribution of the bacterial population of the colon, such that the bifidobacteria become the predominate species, Figure 6 (*38*). Also, lactobacilli may also increase significantly (*39*). The bifidogenic effects of inulin-type fructans are independent of chain length or chemical form (*38,40*), and typically have a dose response effect that appears related to initial bifidobacteria counts (*40-42*).

The bifidogenic effects of inulin-type fructans are well documented in recent reviews (*40,43*) and will not be subject of further elucidation in this paper.

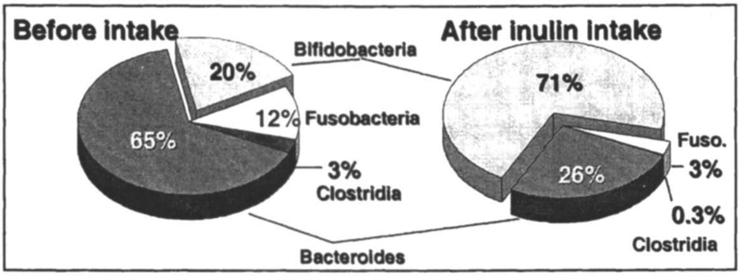

Figure 6. Effect of inulin consumption on colon flora. From data of Gibson et al. (38).

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As colonic microflora utilize the inulin-type fructans for food they produce short chain fatty acids (SCFA)—acetate, propionate, butyrate, resulting in positive influences on local and systemic host health. Other by-products of the fermentation process include bacterial biomass and gas that are eventually excreted. The increase in healthy bacteria biomass results in positive influences on intestinal function by increasing stool bulk and frequency, particularly in constipated patients (*38,39,44-49*). The accompanied increase in the short chain fatty acids, particularly the strong acid acetate, has been shown to lower fecal pH, and help, along with antimicrobials and other substances secreted by the healthy bacteria, to create an environment less competitive for the aforementioned harmful microorganisms (*45,50-53*). A shift in colon flora from predominately proteolytic species to saccharolytic species has association with a reduction in harmful proteolytic products (ammonia, indoles, skatoles, phenols, aliphatic amines, nitrites, cresols, secondary bile acids, and aglycones) (*39,54-57*). Increased amounts of SCFA, in addition to suppressing the colon pH, mediate local growth of the intestinal epithelium, partly as a direct source of energy (butyrate), partly via a stimulation of certain growth hormones (*58,59*). The resulting increased thickness of the intestinal wall in turn reduces the risk of bacterial translocation.

Part of the produced SCFA (mainly acetate and propionate) will be absorbed into the blood stream and delivered to the liver. There they are either used for host energy, creating a caloric value, and/or give rise to specific systemic effects: propionate has been shown in a scientific study to suppress VLDL-triacylglycerol (TAG) synthesis by reducing the activity of *de novo* lipogenic enzymes, and acetate may positively influence the postprandial glucose-response

(*60*). Several human studies have shown the anti-lipogenic effects of inulin-type fructans (*39,61-67*).

Sugar tolerance tests on healthy subjects have further confirmed that inulin-type fructans are not digested or absorbed unlike fructose or glucose, and are safe for diabetics. After the ingestion of sucrose and/or glucose, plasma glucosemic, fructosemic, and insulin effects showed rapid increases, but no corresponding increase was shown with the consumption of inulin-type fructan (42,64). Other research found no influence on serum glucose, no stimulation of insulin secretion and no influence on glucagon secretion (69-71). Inulin has also been shown to reduce postprandial glycemia and insulinemia by 17 and 26%, respectively (*72*). Reduction in fasting insulin levels has also been demonstrated in healthy adults using inulin-type fructan (*66*). In addition, reduced fasting blood glucose has been shown in healthy subjects (*37*) and in diabetic subjects (63) with the use of inulin-type fructans. Inulin-type fructan containing plants have a long history of use to control blood sugar. One of the earliest recorded uses of plants high in inulin as a hypoglycemic agent was by the Greek physician, Theophrastus (*73*). The dandelion is used by various cultures in Eurasia to balance sugar metabolism (*74*), the elecampane root (***Inula helenium***) has been used in North America for this purpose, while the North American Squamish, Kwakawaka'waka, Nuu-chah-nulth and Salish tribes of the Pacific Northwest use camas lily as a sugar replacement (*75*). Inulin also has a long history of use by diabetics (*32,76*) and has been reported to benefit diabetic patients in relatively high doses, above 40 g/day (*77-80*).

Caloric reduction is also a typical attribute of dietary fiber. Inulin-type fructans, as a result of SCFA produced through their fermentation in the colon, provide some metabolic energy as the result of their oxidation in the liver. Based on available caloric studies (*81-84*), combined with specific factors discussed by Roberfroid and others (*5*) the caloric value of 1.5 kcal/g is ascribed for inulin-type fructans.

In addition to their healthy influences in gastrointestinal function and systemic effects on lipid and carbohydrate metabolism, results of animal and human clinical studies have also shown positive effects on mineral absorption, particularly calcium and magnesium, and the potential for cancer prevention in animals. Several studies in animals (*85-95*) and in humans (*96-100*) have shown that absorption of minerals, particularly calcium, magnesium and iron, is enhanced through increased dietary intake of inulin-type fructans. By enhancing the absorption of dietary calcium, inulin-type fructan consumption may contribute to healthier bones and aid in the prevention of osteoporosis, while enhanced absorption of magnesium and iron may also positively influence cardiovascular health.

Different animal studies on the effects of dietary supplementation of inulin-type fructans consistently show a reduced risk in experimentally induced

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carcinogenesis and support continued research on human clinical efforts (*101-115*). The effects were found in both the initiation phase and promotion phase of the carcinogenic process, and thus endorse the statement of *preventive* aspects of inulin-type fructans for this purpose. Moreover, a growth inhibiting effect of inulin-type fructans was found in experimentally implanted tumors in mice, which implies that inulin also has a role in the *treatment* of colon cancer. These early studies and their initial results appear promising and support future research activity.

Significance in Industrial, Academic and Medical Research

Fructans, particularly of the inulin-type, have unique functional and nutritional characteristics that are desirable to meet current world market trends. World trends in food product development have shown that today's consumers require foods that have exceptional taste and eating quality, have low- and/or reduced fat and/or sugar and calories, and can provide health or health-promoting properties or ingredients. In order for these multidimensional products to be effective, they must further be convenient to use and be readily affordable. Unfortnunately, many fiber sources used in the past to produce high fiber foods, have resulted in foods having reduced taste and due to functional limitations, did not provide the diversity required to make most food categories more healthy. Due to recent advances in the production of isolated fructans, the food product developer now has new tools to create healthier high-fiber-containing foods with enhanced organoleptic properties. As the market continues to grow for health-promoting food products, which contain inulin-type fructans, new research opportunities in both nutrition and food science grow. In addition, market focus on the physiological health aspects of fructans as highly functional and unique sources of dietary fiber, will help to communicate further to the consumer that fiber in general is important for their diets. This in turn will educate the public to help increase their fiber consumption.

Due to diets low in fiber and current lifestyle, the average American is now part of the most obese society in the civilized world. As a consequence, we as a society are now experiencing significant increases in chronic diseases having association, based on epidemiological study, with low fiber diets—cancer, coronary disease (CVD), hypertension, gastrointestinal disorders, and type-2 diabetes. Rapid disease intervention and prevention, given the current state of affairs, rather than attempting to find a cure after disease has occurred, indicates timing is now critical for utilizing multidimensional fiber ingredients, such as the fructans, to meet these current consumer health challenges.

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Chapter 12

Yacón' An Ancient Crop in a New Land

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The storage roots of yacón, *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson, are a source of prebiotic fructooligosaccharides that require minimal processing. Yacón has been cultivated for probably more than a thousand years in the Andes, particularly in Peru, Bolivia, Argentina and Ecuador and has been introduced into New Zealand at various times over the past twenty years; however its production as a new, commercial vegetable has not been sustained, so until now it has remained a garden curiosity. The coastal, maritime climate of New Zealand is ideally suited to the cultivation of yacón. Production trials have recorded fresh storage root yields up to 97 tonnes/hectare, which are comparable to those reported in South America, with the dry matter content of the storage roots about 11%. Between 50% and 60% of the dry matter is carbohydrate principally fructans. A major programme of research has commenced to optimise the cultivation, harvesting, storage and processing of yacón tubers. Results of preliminary production, seasonal variation and storage trials are discussed.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

Yacón (*Smallanthus sonchifolius*) (*1*), in the family Asteraceae, is a native crop of the Andes, which is grown principally for its edible underground storage organs (*2*) and has been cultivated, particularly in Peru, Bolivia, northwestern Argentina and Ecuador, for more than a thousand years (*3-4*). Cultural changes in the last century have caused a decline in the cultivation of yacón in South America but recently interest in the carbohydrate chemistry of yacón and in preservation of traditional species diversity has focussed more attention on this crop (*3, 5-6*).

Yacón is an herbaceous plant with aerial stems 27.5-98.4 inches (70-250 cm) in height. The stems are green and purple and are covered with white hairs. The leaves are triangular-ovate and of the sunflower type, Figure 1.

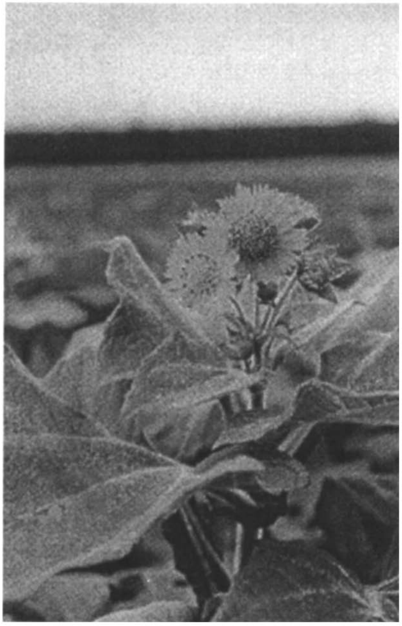

Figure 1. Yacón (Smallanthus sonchifolius).

The yellow flower is relatively small; flowering occurs in autumn after some six months growth and the plant does not often set viable seed. The underground parts, Figure 2, comprise a plant crown, which consists of a tight mass of enlarged, meristematic buds known as turiones, cylindrical roots and swollen storage roots. Most storage roots are 0.22-1.1 lbs (100-500 g), but roots of 2.2 lbs (1 kg) are common and roots of 4.4 lbs (2 kg) have been reported.

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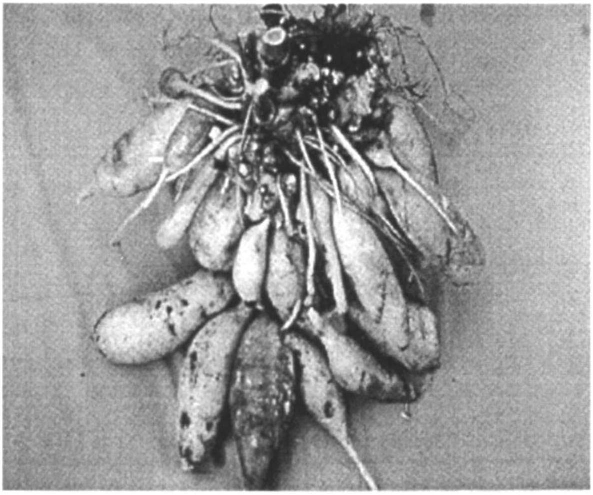

Figure 2. Underground parts of yacón.

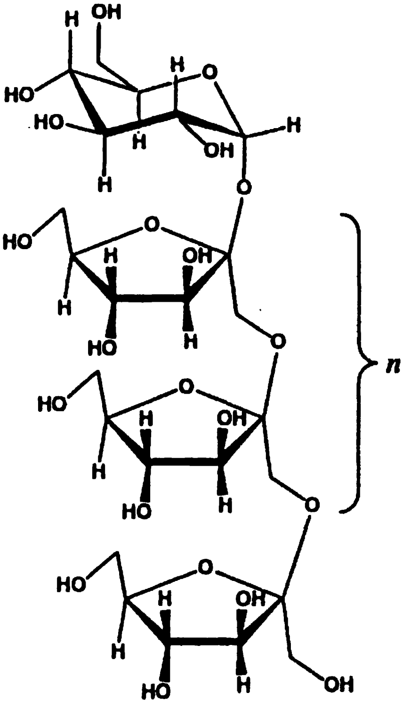

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Figure 3. Structure of inulin.

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Storage Carbohydrates of Yacón

In common with other members of the Asteraceae, the storage carbohydrates are of the inulin type rather than starch. Inulin is a polysaccharide formed by successive additions of β-D-fructofuranosyl units in (1→2)-linkages to sucrose, Figure 3.

Oligosaccharides of the inulin type are commonly known as fructooligosaccharides (FOS) and exhibit prebiotic qualities. A prebiotic is a food that passes undigested through the small intestine of the host animal to the colon where it is selectively fermented by bacteria that are beneficial to the host (*7*). A considerable market therefore exists for fructooligosaccharides, especially in Japan and Europe. Commercially, FOS may be derived by enzymic treatment of sucrose or by greater or lesser degrees of hydrolysis or fractionation of inulin derived from either Jerusalem artichoke (*Helianthus tuberosus*) or chicory (*Cichorium intybus*), which are also members of the *Asteraceae* (*8*). Studies of yacón storage carbohydrates have shown them to be principally fructooligosaccharides rather than the polysaccharide inulin (*9-10*). Yacón thus represents a potential, commercial source of FOS for which minimal processing is required.

Edelman and Jefford (*11*) have described the mechanism for the metabolism of fructans in higher plants; this mechanism permits very rapid changes in the size and number of molecules and hence a change in osmotic potential. During periods of low temperature or drought, rapid depolymerization of fructan occurs to increase osmotic potential and prevent dehydration. Seasonal variations in degree of polymerization occur in yacón (*12*) as well as in Jerusalem artichoke (*13*). In the trial described in reference (*12*) the aerial parts were damaged by typhoon winds in late summer and then regrew. This may have affected the outcome of the trial. Additionally changes in degree of polymerization may occur during post-harvest storage; such changes have been observed for Jerusalem artichoke (*14-16*), chicory (*17-20*) and yacón (*21*).

In addition to a functional food role of the yacón storage roots, the plant has other potential, commercial possibilities. A water extract of the leaves has demonstrated a hypoglycemic effect in diabetes-induced rats (*22*) and an anti-diabetic agent containing sesquiterpenes has been claimed (*23*); in Brazil and Japan the foliage is valued for preparation of an herbal tea (*3*).

Yacón in New Zealand

Background

Yacón was first introduced to New Zealand about twenty years ago (*24*). It has, until recently, remained essentially a garden curiosity although small-scale production in both the North and South Island was reported in 1993 (*25*) and a small field trial was carried out at Crop & Food's Invermay Research Station on the South Island. Recently, more extensive trials have been undertaken in the Waikato and Pukekohe regions (Lat. 37°S) of the North Island with a view to commercializing the production of yacón for domestic and export markets. From New Zealand yacón was introduced to the Czech Republic and Japan and thence to Korea and Brazil (*3*).

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Climatic Requirements

Leaves of yacón are damaged by temperatures below 30°F (-1°C) and are killed almost to ground level by 24 to 26°F (-3 to -4°C). Underground organs are not damaged unless frozen. At Invermay Research Station, Otago (Lat. 45°51'S), a temperature of 19.4°F (-7°C) for several hours damaged all underground organs (*25*). Prolonged periods of high temperature can have a negative effect upon vegetative growth and storage roots.

The New Zealand climate is temperate and dominated by a westerly wind flow within an oceanic environment, which gives a weather pattern that is changeable over short periods. The Waikato region has an annual rainfall of 47 inches (1200 mm) spread evenly throughout the year. The mean summer temperature (January) is 64°F (17.8°C) and the winter temperature (July) 47°F (8.3°C) with an average of 228 days in a growing season without air frosts. Summer air temperatures above 86°F (30°C, measured in a shaded screen) are uncommon. Ground frosts may occur in winter down to 16°F (-9°C) but without snow and ground freezing. Thus, the climate of the Waikato region is ideally suited for the cultivation of yacón.

Preliminary Trial (2000-2001)

In preliminary trials (2000-2001), root crops matured in 6-7 months after a spring planting of a crown piece with a density of 9000 plants/acre (22200 plants/hectare). Separations were 4.9 feet (1.5m) between rows and 12 inches (30 cm) within rows. The root crop was harvested in early winter (June) when the top growth was killed by frost. Storage roots, which were left in the ground unharvested, kept well for several months.

Root yields of up to 42 tons/acre (96 tonnes/hectare) were obtained with individual plants producing as much as 8.8lbs (4 kg) of roots and with individual roots up to 2.2lbs (1 kg) in weight. The dry matter content of the storage roots varied from 10.2% to 11.7% with an average of 11% for four different accessions. In a parallel trial, the root yield of Jerusalem artichoke tubers was 22.8 tons/acre (50 tonnes/hectare) with an average dry matter yield of 13.4%. The fresh root yields of yacón are consistent with those obtained in South America (*26, 27*).

Pests

It is reported (*3*) that yacón is affected by a wide range of insect pests, but only a few bacterial and fungal diseases have been noted in South America and Japan. In New Zealand, there have been no diseases of importance in the field crops. In postharvest storage, Rolf's disease (*Athelia rolfsii*) caused rotting of unwashed crowns stored in bulk piles covered with sawdust in the field. This disease can be overcome by washing the crowns prior to storage and greater attention to hygienic storage conditions. The disease can also be controlled by treating cleaned, diseased crowns by dipping them in liquid fungicide such as Rizolex™ (tolclofosmethyl) prior to planting. The major pests of yacón have been caterpillars grazing the foliage in summer and

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autumn; the principal species identified was Soyabean looper (*Thysanaplusia orichalcea*). However green looper (*Chrysodeixis eriosoma*) and tomato fruitworm (*Helicoverpa armigera*) have also been present, but in lower numbers. White fly (*Trialeurodes vaporariorum*) may affect outdoor crops under favorable conditions, but it is more likely to be a significant pest if yacón is grown in a greenhouse. These insects are well controlled by insecticides such as Steward™ (indoxacarb), Monitor™ (methamidophos) or Lannate™ (methomyl).

Seasonal Variations Trial (2001-2002)

In order to optimize fructooligosaccharide content at harvesting, a second trial was commenced in 2001-2002. In this trial, the storage roots were sampled every 25 days commencing at 5 months after planting, which occurred in October 2001. Four replicates were collected and each replicate consisted of two storage roots from each of two plants. Only roots in excess of 8.8oz (250 g), fresh weight, were selected. Roots were extracted, in triplicate, using the method described by Jaime *et al.* (*28*) for determination of total fructan and fructooligosaccharides in onion tissue. Extracted material was analyzed by HPLC using Shodex KS801 and KS802 columns in series eluted with water at 1 mL/min at 50°C. Detection was by refractive index. The exclusion limit was 1 x 10^3 corresponding to ~6 glycosyl units and none of the fructan material was excluded, which indicated that it was all oligosaccharides.

A typical chromatogram of a mature storage root from the 2000-2001 trial is shown in Figure 4. This root was deep frozen before freeze drying and extraction. The unlabeled peak (9.3 min) in Figure 4 shows UV absorption presumed consistent with the presence of, *inter alia*, phenolics. The presence of phenolics was inferred from the strong darkening of fresh storage root material upon maceration. Antioxidants including tricaffeoylaldaric acid have been isolated from yacón storage roots (*29, 30*). Figure 5 shows an HPLC chromatogram of the extract of a 5 month storage root for comparison. The relatively large quantities of lower degree of polymerization (DP) fructooligosaccharides, including 1-kestose, and of glucose in the immature root indicate that fructooligosaccharides are being elaborated. In contrast the larger quantities of fructose in the mature root appear to indicate that some degree of depolymerization had occurred, probably as a result of onset of cooler climatic conditions.

Results for the seasonal variation trial are shown in Figure 6. Total extractable carbohydrate content varied between 76 and 83% composition on a dry matter basis (DMB).

Dry matter, total extractable carbohydrates and fructooligosaccharides showed a maximum on 25 May (7 months after planting), at which time flowering had commenced. The plants were the same age as observed by Fukai *et al.* (*12*) in an equivalent study in the Northern hemisphere. However the planting took place one month earlier in the season (October) for this study compared with the equivalent month in the Northern hemisphere study (May) (*12*). The similarity is remarkable

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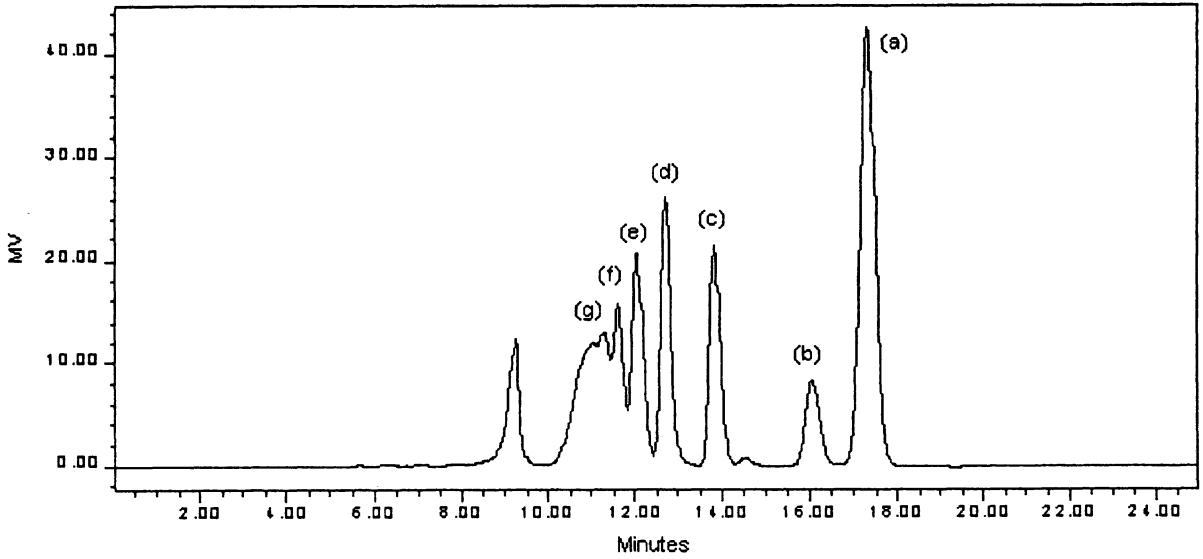

Figure 4. HPLC chromatogram of extract of mature yacón storage root showing fructose (a), glucose (b), sucrose (c), 1-kestose (d), nystose (e), kestapentaose (f), fructooligosaccharides DP 6+ (g). Conditions as described in the text.

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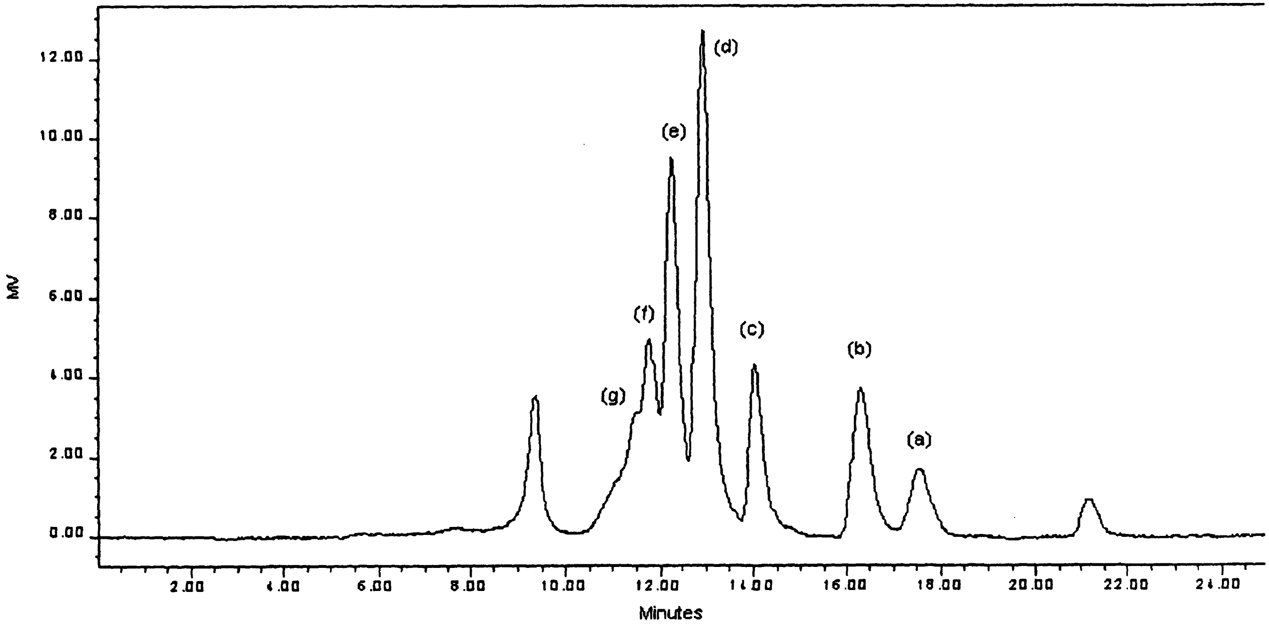

Figure 5. HPLC chromatogram of immature (5 month) yacón storage root; (a)-(g) as for Figure 4. Conditions as described in the text. The peak at 21.4 minutes is residual ethanol.

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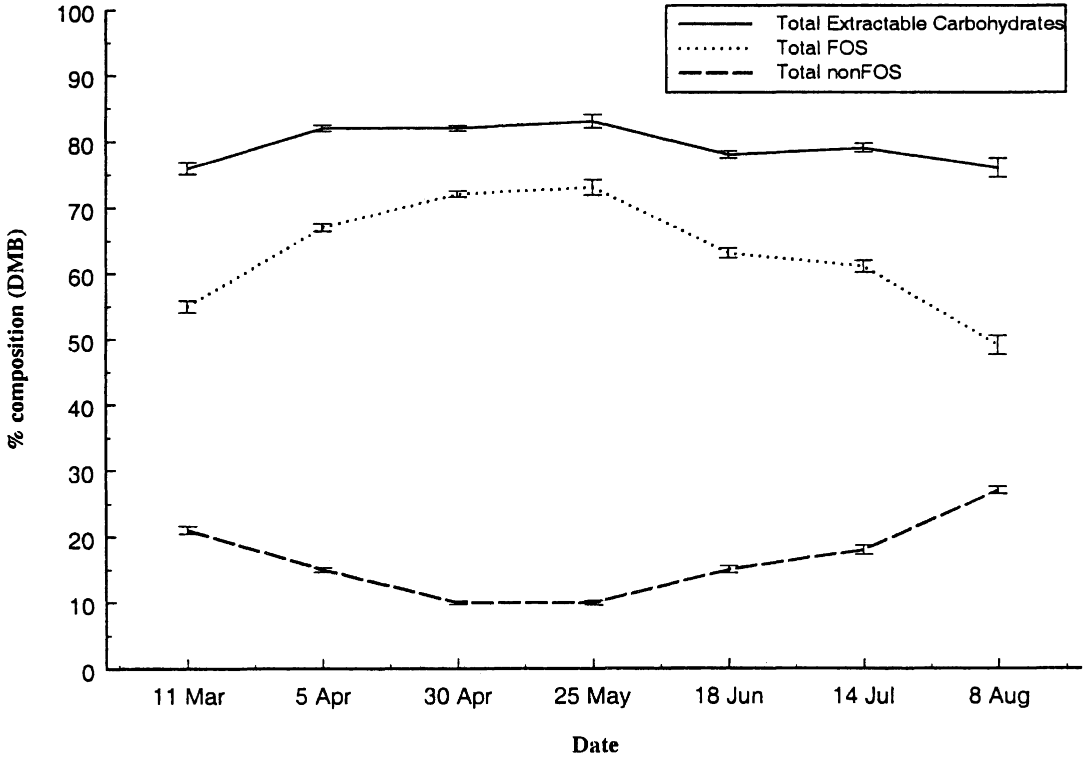

Figure 6. Seasonal variation in total extractable carbohydrates, total fructooligosaccharides (FOS) and combined total of glucose, fructose and sucrose (nonFOS).

since in reference (*12*) a typhoon damaged the aerial portions of the plants some 4 months after planting. As expected, regression analysis showed that there was a strong, negative, linear correlation ($R^2 = 0.85$) between total fructooligosaccharide content and total combined content of glucose, fructose and sucrose (*11*).

Storage Trials 2002

Storage trials commenced in August using material sampled 10 months after planting. It is apparent from the seasonal variation trial, that this was not the optimum time for harvest, if the intention was to maximize FOS content. When the trial commenced, samples were stored at two temperatures, 50°F (10°C) and 41°F (5°C). Before the trial commenced, storage roots were examined carefully to ensure that they were undamaged, clean and dry. By the end of the first week of the trial, the roots, which were stored at the lower temperature, showed extensive splitting and by week 4 the majority were decomposed. Cisneros-Zevalos *et al.* (*31*) were able to complete a trial of 90 days at 4°C with 17, 21 and 27% loss for different accessions. In contrast, Asami *et al.* (*21*) observed a 33% decrease in FOS after only two weeks storage at 5°C. It is possible that both the accession and the time of harvesting affect storage behavior at lower temperatures.

The results for the 50°F (10°C) trial are shown in Figure 7. For the first week FOS levels remained stable but after 14 days there was a 16% decrease in FOS content and at 28 days the decrease was 23%. These results fall between the 25°C and 4°C storage trials of reference (*31*).

Conclusions

At publication the study was still ongoing. The results to date indicate that yacón is a suitable crop for cultivation in New Zealand and no egregious pests were found. As expected (*11*) there was a negative correlation between the FOS content and the mono/disaccharide content. The former is optimal for harvesting some 7 months after planting. Storage trial results show disparity with the literature (*21, 31*). However there are also inconsistencies within the literature. In this study, storage at a temperature of 50°F (10°C) retained FOS levels for 1 week without loss and for 4 weeks with a decrease of 23%.

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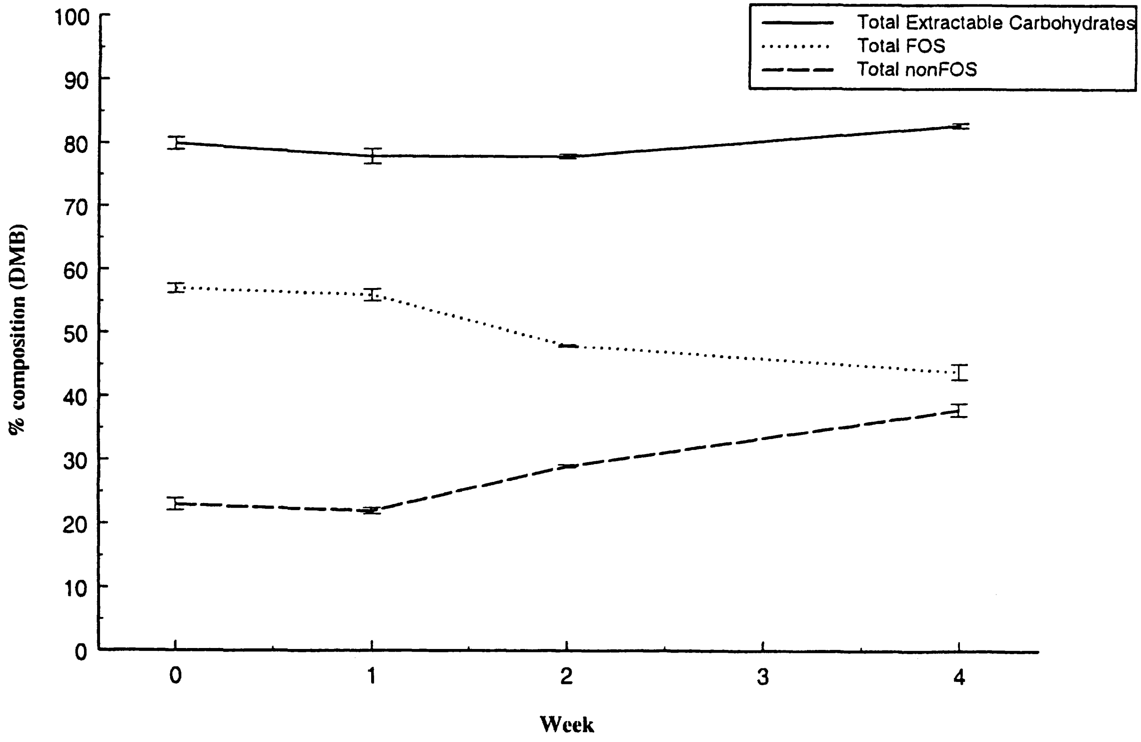

Figure 7. Results of storage trial at 50 °F (10 °C) for total extractable carbohydrates, total fructooligosaccharides (FOS) and combined total of glucose, fructose and sucrose (nonFOS).

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Chapter 13

Enzymatic Synthesis of Acarbose Oligosaccharide Analogues as New Enzyme Inhibitors

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Acarbose, a pseudotetrasaccharide and inhibitor for several carbohydrases, was modified at the reducing-end by *Bacillus stearothermophilus* maltogenic amylase transglycosylation reactions with several mono- and disaccharide acceptors to give new, specific, and more potent carbohydrase inhibitors. Acarbose was also modified at the nonreducing-end by *B. macerans* cyclomaltodextrin glucanyltransferase transglycosylation reactions between cyclomaltohexaose and acarbose. These analogues have been found to be very potent and specific inhibitors for porcine pancreatic, *Aspergillus oryzae,* and *Bacillus amyloliquefaciens,* α-amylases with K_I values of 10, 30, and 40 nM, respectively.

Introduction

Enzymes are nature's catalysts. Besides being proteins, they differ from ordinary catalysts by having a high degree of specificity and often only catalyze one, or at the most, only a few specific reactions, using specific substrates (reactants) that have particular features in common. They give rise to specific products that also have common features. Enzymes are increasingly being used in industry to produce new and specific products that cannot be made by conventional methods.

In the present study, we have used two types of enzymes, *Bacillus stearothermophilus* maltogenic amylase (BSMA) and *Bacillus macerans*

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

cyclomaltodextrin glucanyltransferase (CGTase), to modify acarbose. Acarbose is a natural product produced by various strains of *Actinoplanes*. It is a pseudo tetrasaccharide with an unsaturated cyclitol [2,3,4-trihydroxy-5-(hydroxymethyl)-5,6-cyclohexene in a D-*gluco*-configuration] attached to the nitrogen of 4-amino-4,6-dideoxy-D-glucopyranose, which is linked α-1→4 to maltose (see Fig. 1). Acarbose is a strong competitive inhibitor of α-glucosidase (*1–3*), which has led to its use in the treatment of diabetes mellitus [4]. It has also been shown to be an inhibitor of α-amylases (*1–3,5*), cyclomaltodextrin glucanyltransferase (*3,6,7*), glucoamylase (*8,9*) and glucansucrases (*10,11*).

The enzymatic synthesis of a wide variety of oligosaccharides has been obtained *in vitro* by transfer reactions between a segment of a donor and various kinds of acceptors. Very often, the transfer takes place from a specific donor to a relatively large number of acceptors that have different structures. The specificity of the transfer depends on the specific enzyme used, which usually determines the position and configuration of the glycosidic bond that is formed. The structure of the acceptor also is often important in determining the position of the transfer (*12,13*).

Modification of Acarbose by *B. stearothermophilus* Maltogenic Amylase

B. stearothermophilus maltogenic amylase (BSMA) reacted with acarbose to cleave the first glycosidic linkage, giving D-glucose and acarviosine-D-glucose (*14*) (see A in Fig. 1). During the course of the reaction, a new acarbose analogue was observed, which was a transfer product in which acarviosine-D-glucose was transferred to C-6 of D-glucose to give an α-1→6 linked acarbose analogue (4-α-acarviosine-4-α-D-glucopyranosyl isomaltose) that we called *isoacarbose* (*15*) (see B in Fig. 1).

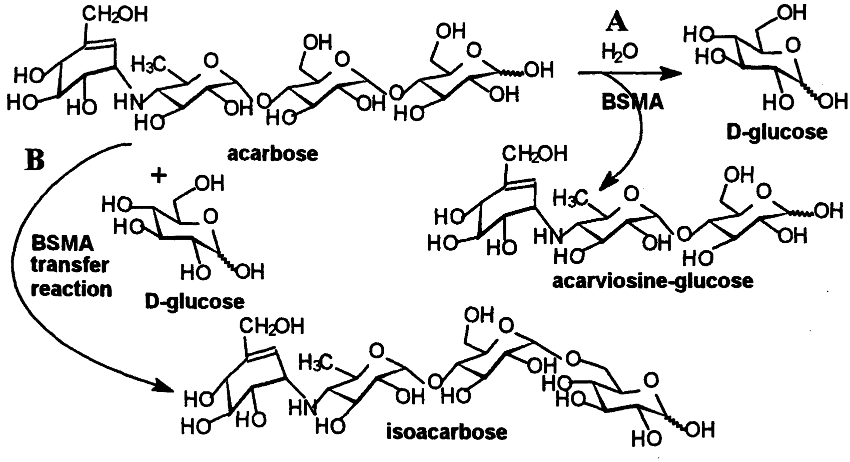

*Figure 1 Acarbose reactions with BSMA: **A**, hydrolysis to give acarviosine-glucose + D-glucose; **B**, transfer reaction with D-glucose to give isoacarbose.*

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We then tested a number of monosaccharide acceptors such as D-glucose, D-mannose, D-galactose, D-fructose, D-xylose, methyl-α-D-glucopyranoside and several disaccharides, such as maltose, cellobiose, lactose, gentiobiose, α,α-trehalose, sucrose, raffinose, and maltotriose (*15*). The acceptor products were prepared by using a 10% (w/v) solution of acarbose and 20% (w/v) solution of acceptor in 50 mM citrate buffer (pH 6.0). The mixture was incubated 1 h at 55 °C and the reaction was initiated by the addition of 10 IU of BSMA per mg of acarbose (1 IU = the amount of enzyme necessary to transfer 1 μmole of acarviosine-glucose to acceptor in 1 min.). The reaction was carried out for 24 h at 55 °C. The products of the reaction were analyzed by thin-layer chromatography (TLC) on Whatman K6 plates, using 2 ascents of 1:3:1 volume proportions of ethylacetate/2-propanol/water. The plate was dried and visualized by dipping into 0.3% (w/v) N-(1-naphthyl)-ethylenediamine, 5% (v/v) H_2SO_4 in methanol, followed by heating at 120 °C for 10 min. See Figs. 2 & 3.

We purified several of the new acceptor products that resulted by descending paper chromatography, gel-permeation chromatography on Bio-Gel P2, and flash chromatography on silica gel. The structures of the various acceptor products were then determined by specific enzyme reactions, followed by TLC analysis, methylation analysis, and ^{13}C-NMR (*15*). The structures for the various acceptor products are shown in Fig. 4. The predominant linkage that was formed in the transglycosylation reaction from acarbose to an acceptor was the α-1→6 linkage. There also, however, was some α-1→3 linkages and in a few cases some α-1→4 linkages that were formed as well.

The D-glucose acceptor product gave the transfer of acarviosine-glucose to the C-6 position of the D-glucose acceptor, giving acarviosine linked α-1→4 to isomaltose and the formation of isoacarbose (4^{II}-α-acarviosine-isomaltose). The acceptor reaction with maltose gave three products of which the major product was acarviosine-glucose linked α-1→6 to the nonreducing-end of maltose. This compound is named 4-α-acarviosine-6^{II}-α-D-glucopyranosyl maltose. The first minor product was acarviosine-glucose linked α-1→3 to the nonreducing-end of maltose, giving 4-α-acarviosine-3^{II}-α-D-glucopyranosyl maltose and the second minor product was acarviosine-glucose linked α-1→4 to the nonreducing-end of maltose, giving 4-α-acarviosine-4^{II}-α-D-glucopyranosyl maltose or 4^{III}-α-acarviosine maltotriose.

The major acceptor products for cellobiose and lactose were the transfer of acarviosine-glucose to the C-6 positions to give 4-α-acarviosine-6^{II}-α-D-glucopyranosyl cellobiose and 4-α-acarviosine-6^{Gal}-α-D-glucopyranosyl lactose. Sucrose gave two acceptor products, 4-α-acarviosine-4^{Glc}-α-D-glucopyranosyl sucrose and 4-α-acarviosine-6^{Glc}-α-D-glucopyranosyl sucrose in about equal amounts. Gentiobiose gave a single product that had acarviosine-glucose linked to C-6 of the nonreducing-end D-glucose residue to give 4-α-acarviosine-6^{II}-α-D-glucopyranose gentiobiose. α,α-Trehalose gave two products in about equal

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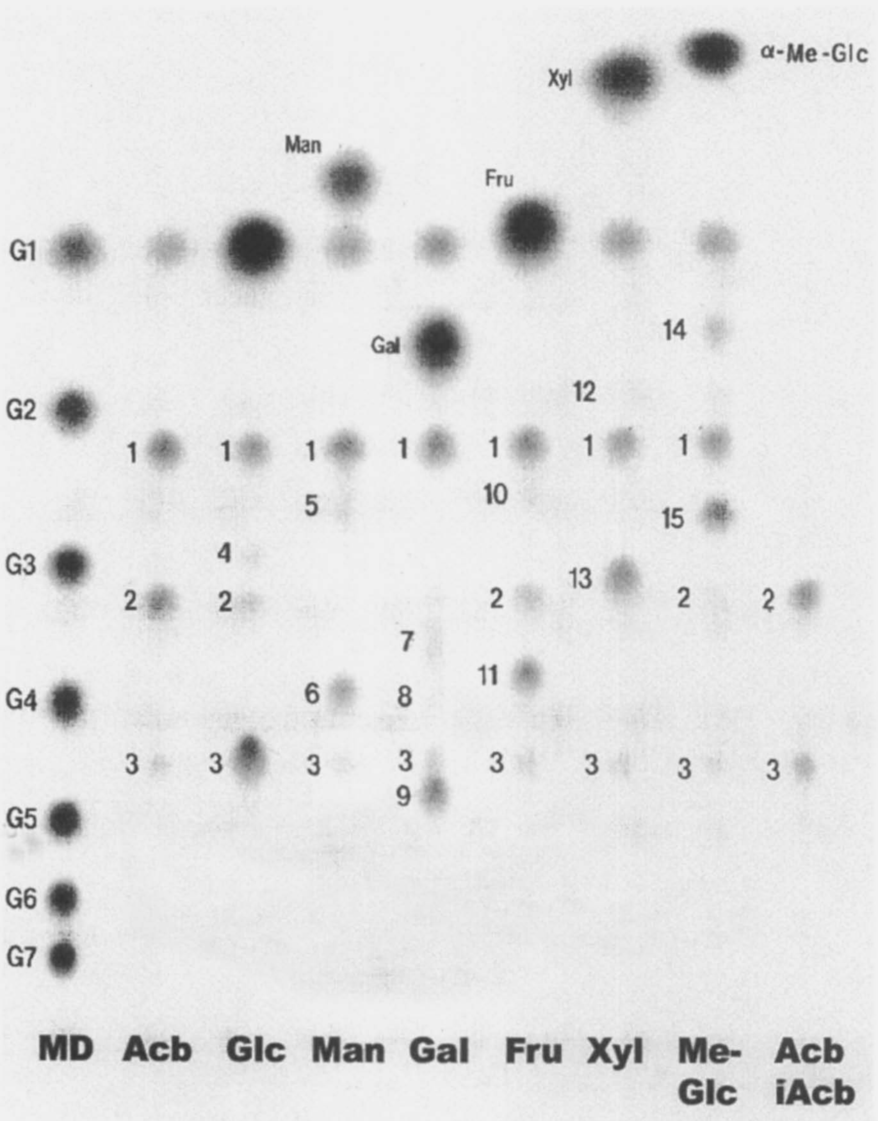
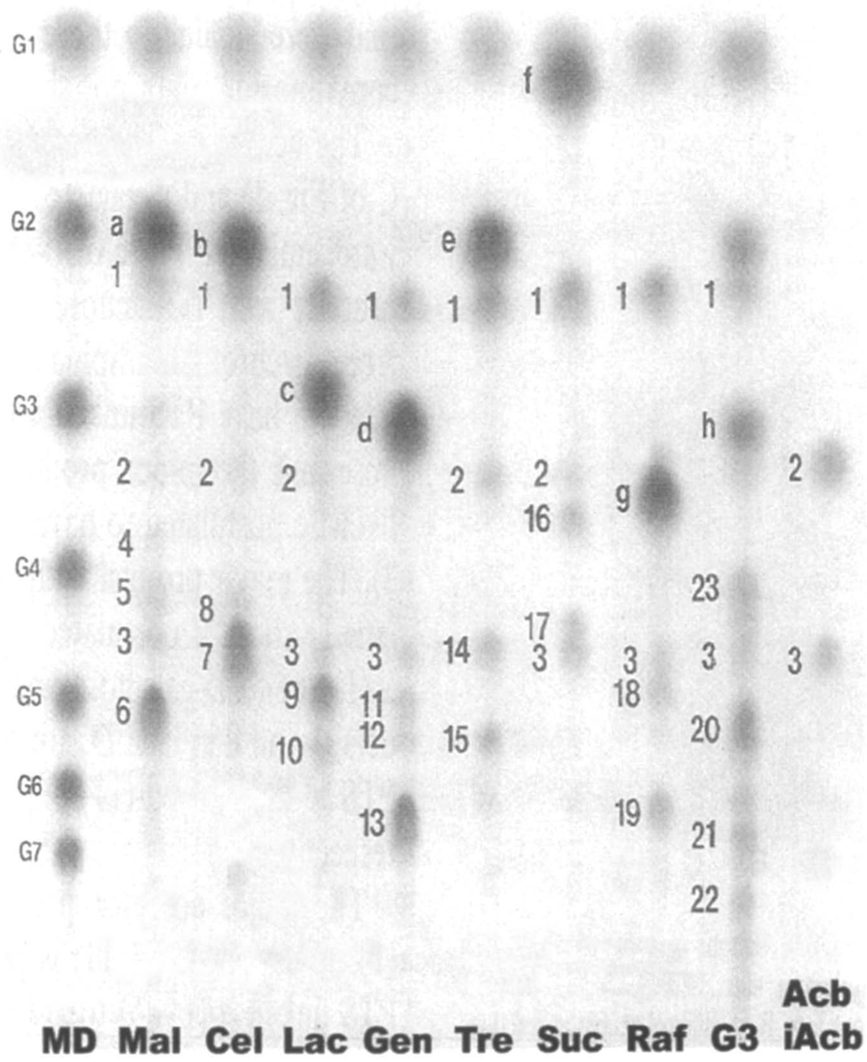

α-Me-Glc
Xyl
Man
Fru
G1
Gal
14
12
G2
1
1
1
1
1
1
1
5
10
15
G3
4
13
2
2
2
2
2
7
11
G4
6
8
3
3
3
3
3
3
3
3
9
G5
G6
G7
MD
Acb
Glc
Man
Gal
Fru
Xyl
Me-Glc
Acb iAcb

Figure 2. TLC of monosaccharide acceptors. 1= acarviosine-glucose; 2= acarbose; 3 = isoacarbose; 6 = Man product; 9 = Gal product; 11 = Fru product; 13 = Xyl product; 14 & 15 = Me-α-Glc product; 4 & 5, 7 & 8, and 10 – 12 = very minor unidentified products. (Reproduced with permission from reference 15. Copyright 1998 Elsevier Science.)


G1
G2
G3
G4
G5
G6
G7
a
b
c
d
e
f
g
h
1
2
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4
5
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14
15
16
17
18
19
20
21
22
23
MD
Mal
Cel
Lac
Gen
Tre
Suc
Raf
G3
Acb
iAcb

Figure 3. TLC of di- & tri-saccharide acceptors. A-h are the respective acceptors. 1,2,3 = same as in Fig. 2; 6 = maltose product; 7 = cellobiose product; 9 = lactose product; 13 = gentiobiose product; 14 & 15 – α, α -Tre product; 16 & 17 = Suc product; 18 & 19 = Raf product; 20-22 = G3 product; 4 & 5, 8, 10-12 = very minor unidentified products. (Reproduced with permission from reference 15. Copyright 2002 Elsevier Science.

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amounts in which acarviosine-glucose was linked to the C-6 and to C-4 positions to give 4-α-acarviosine-6-α-D-glucopyranosyl-α,α-trehalose and 4-α-acarviosine-4-α-D-glucopyranosyl-α,α-trehalose in about equal amounts. Raffinose also gave two products in which acarviosine-glucose was linked to the C-6 and to C-4 positions of the D-galactose moiety of raffinose, with the former in about twice as much as the latter. See Fig. 4 for the structures of these acarbose analogues.

Inhibition Studies of Reducing-end Modified Acarbose Oligosaccharide Analogues

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Acarbose, acarviosine-glucose (4-α-acarviosine-D-glucopyranose), and isoacarbose (4^{II}-α-acarviosine isomaltose) were competitive inhibitors of α-glucosidase and mixed competitive inhibitors of porcine pancreatic α-amylase and *B. macerans* cyclomaltodextrin glucanyltransferase (*16*). Acarviosine-glucose was a potent inhibitor for baker's yeast α-glucosidase, inhibiting 430-times more than acarbose, but was only 40% as good as acarbose for rat intestine α-glucosidase. Acarviosine-glucose was an excellent inhibitor for cyclomaltodextrin glucanyltransferase, inhibiting 6-times more than acarbose. Isoacarbose was the most effective inhibitor for α-amylase and cyclomaltodextrin glucanyltransferase, inhibiting 15- and 2-times more than acarbose, respectively. Table 1 gives the type of inhibition, the K_I values, and the relative inhibitor potency for the acarbose analogues with different enzymes.

The cellobiose and lactose acarbose acceptor products (4-α-acarviosine-6^{II}-α-glucopyranosyl cellobiose and 4-α-acarviosine-6^{Gal}-α-D-glucopyranosyl lactose) were both found to be potent competitive inhibitors for β-glucosidase, with K_I values of 0.45 μM and 0.52 μM, respectively, and acarbose was not an inhibitor at all (*17*). The lactose analogue was a good uncompetitive inhibitor for β-galactosidase, with K_I value of 159 μM, while acarbose was not an inhibitor at all. Both the cellobiose and the lactose analogues were also potent mixed inhibitors for cyclomaltodextrin glucanyltransferase, with K_I values of 0.8 μM and 1.2 μM with potencies of 3.2- and 2.2-times that of acarbose. The type of inhibition, the K_I values, and the relative inhibitor potencies are given in Table 1.

The cellobiose analogue could be a potent inhibitor for cellulase and cellobiohydrolase. The α-α-trehalose analogues could be potent inhibitors of trehalases; the gentiobiose analogue could be a potent inhibitor for β-1→6 hydrolases; and the sucrose analogues might be inhibitors for invertases. Isoacarbose could be a potent inhibitor for dextranase. To date, these analogues have not been tested as inhibitors for these enzymes.

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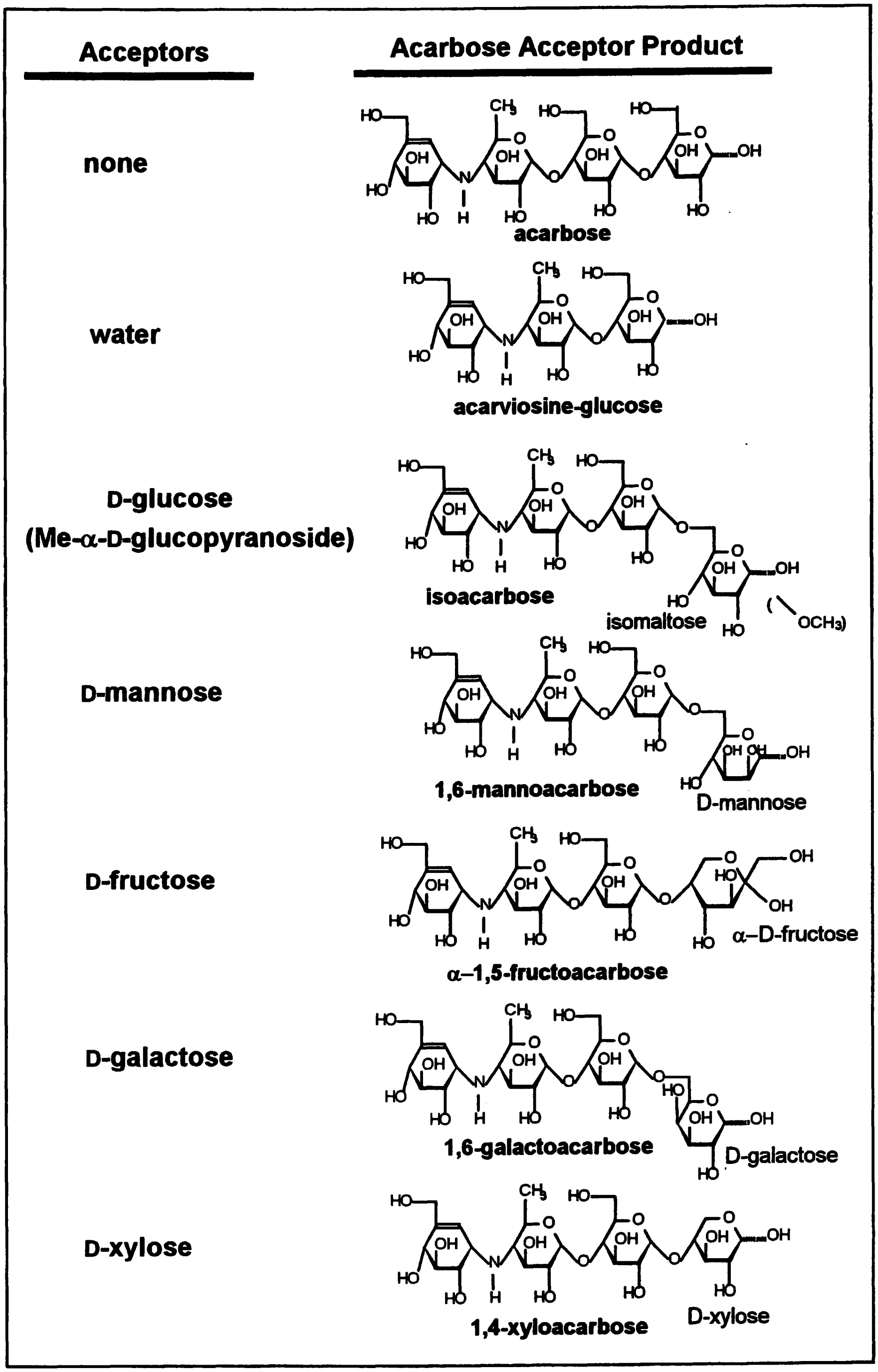
Acceptors
Acarbose Acceptor Product
none
acarbose
water
acarviosine-glucose
D-glucose
(Me-α-D-glucopyranoside)
isoacarbose
isomaltose
(OCH3)
D-mannose
1,6-mannoacarbose
D-mannose
D-fructose
α-1,5-fructoacarbose
α-D-fructose
D-galactose
1,6-galactoacarbose
D-galactose
D-xylose
1,4-xyloacarbose
D-xylose

Figure 4 Structures of Acarbose Monosaccharide Acceptor Analogues

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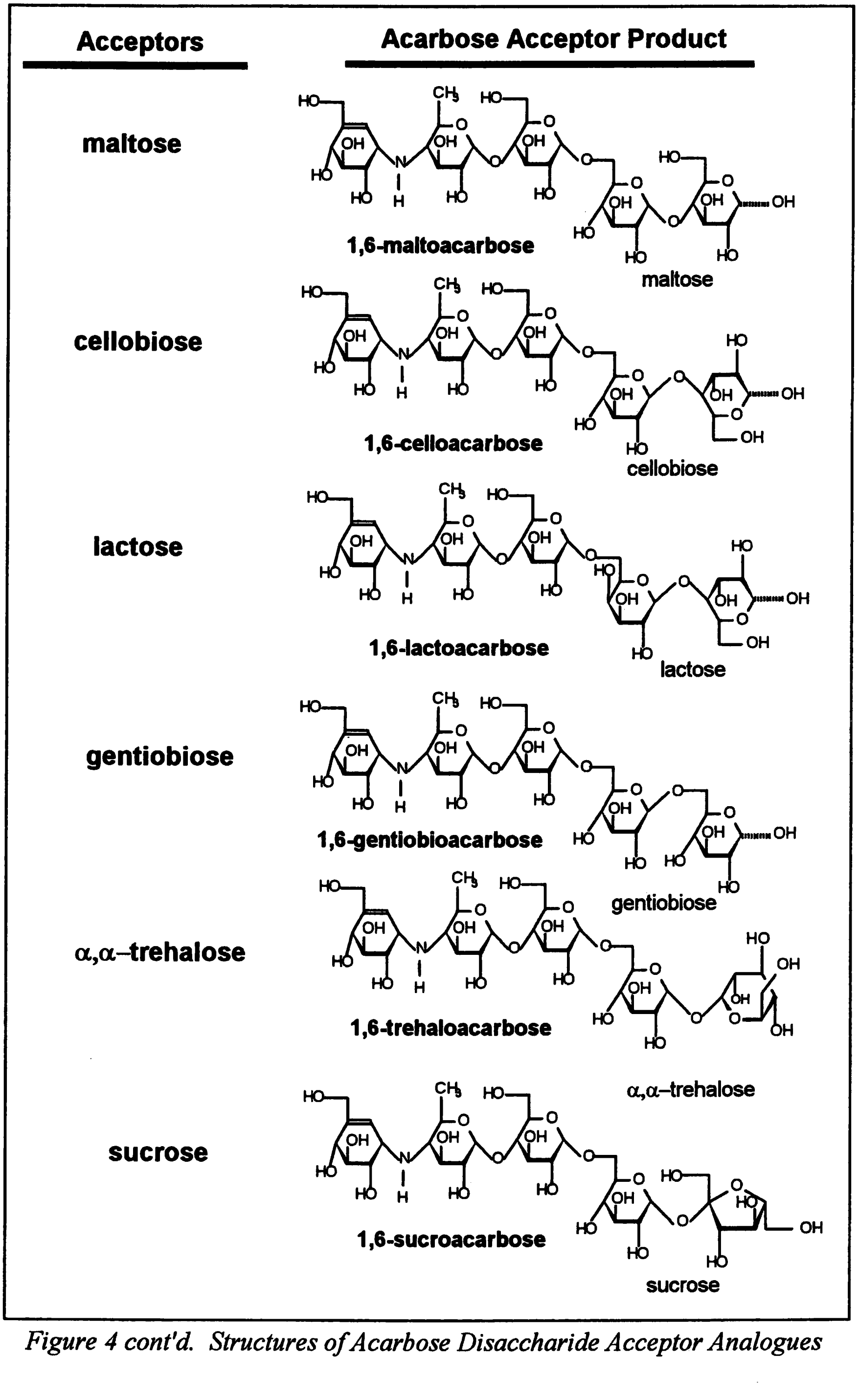
Acceptors
Acarbose Acceptor Product
maltose
1,6-maltoacarbose
maltose
cellobiose
1,6-celloacarbose
cellobiose
lactose
1,6-lactoacarbose
lactose
gentiobiose
1,6-gentiobioacarbose
gentiobiose
α,α–trehalose
1,6-trehaloacarbose
α,α–trehalose
sucrose
1,6-sucroacarbose
sucrose

Figure 4 cont'd. Structures of Acarbose Disaccharide Acceptor Analogues

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Table I. Inhibition of Enzymes by Acarbose Reducing-end Analogues

Enzymes	*Inhibitors*	*Type of Inhibition*	*K_I (μM)*	*Potency*[a]
Baker's yeast α-glucosidase	Acarbose	Competitive	78	1.0
	Acarviosine-Glc	Competitive	0.18	430
	Isoacarbose	Competitive	405	0.2
	6^{II}-α-AGLac	Competitive	12.3	6.3
Rat intestine α-glucosidase	Acarbose	Competitive	0.60	1.0
	Acarviosine-Glc	Competitive	0.13	4.6
	Isoacarbose	Competitive	0.13	4.6
CGTase	Acarbose	Mixed	2.53	1.0
	Acarviosine-Glc	Mixed	0.40	6.3
	Isoacarbose	Mixed	1.25	2.0
	6^{II}-α-AGCel[a]	Mixed	1.20	2.1
	6^{II}-α-AGLac[a]	Mixed	0.80	7.2
Pig Pancreas α-Amylase	Acarbose	Mixed	0.64	1.0
	Acarviosine-Glc	Mixed	0.54	1.2
	Isoacarbose	Mixed	0.02	15.2
β-glucosidase	Acarbose	None	—	—
	6^{II}-α-AGCel[b]	Competitive	0.45	—
	6^{II}-α-AGLac[b]	Competitive	0.52	—
β-galactosidase	Acarbose	None	—	—
	6^{II}-α-AGLac[b]	Uncompetitive	159	—
	Isoacarbose	Competitive	430	—

^{Potency} is defined relative to that of acarbose.

[b] 6^{II}-α-AGCel is the acarviosine-glucosyl-cellobiose acarbose analogue and 6^{II}-α-AGLac is the acarviosine-glucosyl-lactose acarbose analogue.

Addition of Maltodextrins to the Nonreducing-end of Acarbose by the Reaction of Cyclomaltohexaose and *Cyclomaltodextrin Glucanyltransferase*

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Acarbose was modified at the other end, the nonreducing-end, by the reaction of acarbose with cyclomaltohexaose and *cyclomaltodextrin glucanyltransferase* (CGTase). Three major CGTase coupling products (P1, P2, and P3) were formed sequentially (see Fig. 5). The three products were purified by Bio-Gel P2 gel-permeation chromatography (see Fig. 6). Digestion of the products by β-amylase and glucoamylase showed that they were composed of maltohexaose (G6), maltododecaose (G12), and maltooctadecaose (G18), respectively, attached to the nonreducing-end of acarbose (*18*).

The type of linkage between the maltodextrins and acarbose was determined by ^{13}C-NMR of the glucoamylase product, D-glucopyranosyl acarbose, in which it was found that there was a significant downfield chemical shift of C-4 of the 5-hydroxymethyl cyclohexene ring of 5.2 ppm, indicating that the D-glucopyranosyl unit was attached to the C-4-OH of the 5-hydroxymethyl cyclohexene ring of acarbose, and that the linkage was α- because of the formation of a peak at 98.5 ppm for the anomeric carbon of the D-glucopyranosyl unit, which is characteristic of an α-linkage (*18, 19*). The maltodextrins of the three products are, thus, linked α-1→4 to the nonreducing-end of acarbose.

The migration of the three products on TLC, their digestion by β-amylase and glucoamylase, and the ^{13}C-NMR showed that P1 had maltohexaose (G6) attached α-1→4 to the nonreducing-end of acarbose to give 4^{IV}-α-maltohexaosyl acarbose (G6–A); P2 had maltododecaose (G12) attached α-1→4 to the nonreducing-end of acarbose to give 4^{IV}-α-maltododecaosyl acarbose (G12–A); and P3 had maltooctadecaose (G18) attached α-1→4 to the nonreducing-end of acarbose to give 4^{IV}-α-maltooctadecaosyl acarbose (G18–A). P1 was formed by the reaction of acarbose with α-CD to give (G6–A); P2 was formed by reaction of P1 (G6–A) with α-CD to give (G12–A); and P3 was formed by reaction of P2 (G12–A) with α-CD to give (G18–A). There, thus, resulted a pattern of three products in which the number of D-glucose residues in the maltodextrin chains differed by multiples of six. The minor products observed in the later stages of reaction (Fig. 6) were most probably produced by disproportionation reactions between the three coupling products (P1 + P1), (P1 + P2), (P1 + P3), (P2 + P3), and (P3 + P3). The relatively low amounts of these minor products indicate that the coupling reactions were highly favored over the disproportionation reactions, even after a long reaction time of 144 h. This is interpreted by postulating that α-CD is the favored compound bound at the active-site of CGTase, which opens the cyclomaltohexaose ring to give maltohexaose (G6) covalently linked at the active-site (*20*). Acarbose then reacts with this covalent

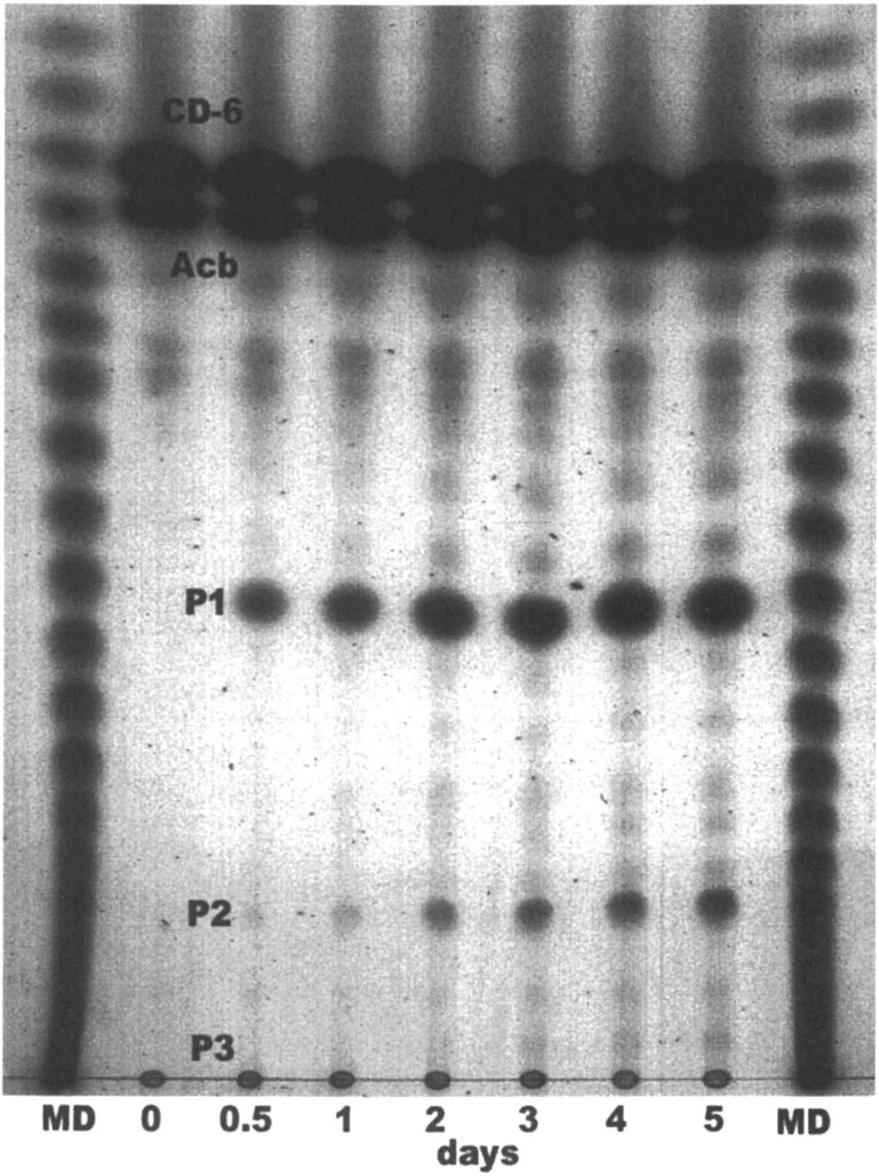

Figure 5. TLC (Whatman K5 (10x20cm) developed 2-times (18 cm path length) with 85:20:50:70 parts by vol of $CH_3CN/EtOAc/1\text{-}PrOH/H_2O$) of the CGTase reaction products from α-CD and acarbose. Lanes 1 & 9 = maltodextrin standards; lanes 2-8 = CGTase reaction products at 0, 0.5, 1, 2, 3, 4, and 5 days.

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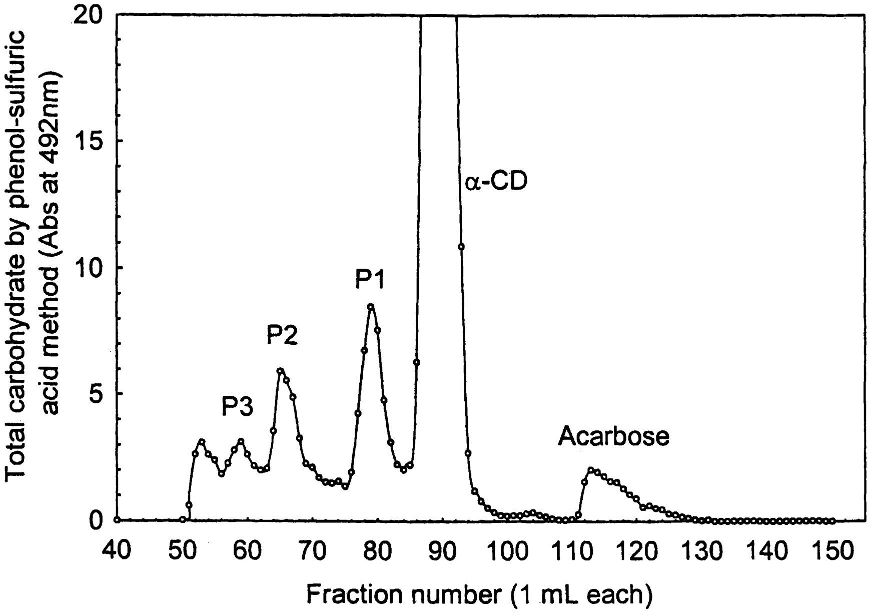
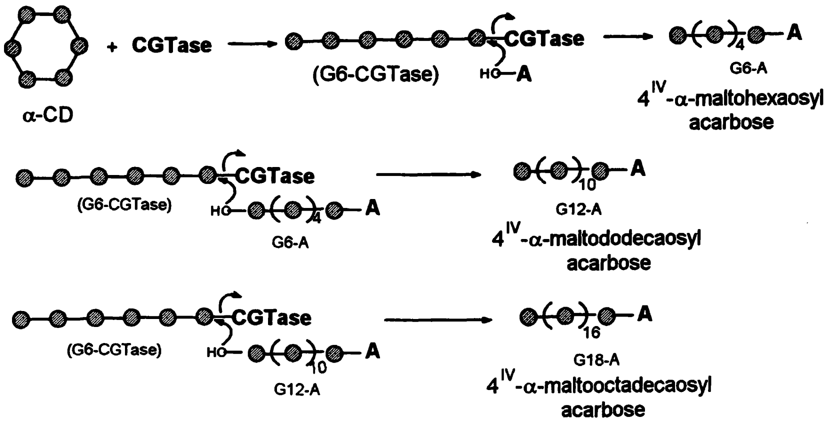


Figure 6. BioGel P-2 column (1.5 cm x 100 cm; flow rate 0.6 mL/10min) chromatography of CGTase reaction products from cyclo-maltohexaose and acarbose. (Reproduced with permission from reference 18. Copyright 2002 Elsevier Science.)

CGTase complex to give the transfer of G6 to the nonreducing-end of acarbose. The nonreducing-end of G6-acarbose can then react with the G6-enzyme complex to give G12-acarbose, which in turn can react with the G6-enzyme complex to give G18-acarbose (see Fig. 7).

These 4^{IV}-maltodextrinyl-acarbose analogues have been found to be the most potent inhibitors, so far known, for α-amylases. Porcine pancreatic, *Aspergillus oryzae,* and *Bacillus amyloliquefaciens* α-amylases had K_I values of 10, 30, and 40 nM, respectively.



+ CGTase
α-CD
(G6-CGTase)
CGTase
HO—A
G6-A
4^{IV}-α-maltohexaosyl acarbose
(G6-CGTase)
G6-A
G12-A
4^{IV}-α-maltododecaosyl acarbose
(G6-CGTase)
G12-A
G18-A
4^{IV}-α-maltooctadecaosyl acarbose

Figure 7 Proposed mechanism for the addition of maltodextrin chains, containing multiples of six D*-glucose residues, to the nonreducing-end of acarbose by the reaction of acarbose with cyclomaltohexaose (a-CD) and cyclomaltodextrin glucanyltransferase (CGTase). Circles represent* D*-glucose residues and A represents acarbose. Reprinted from reference 18 by permission of Elsevier Science.*

In summary, we have shown enzymatic modifications of the reducing-end and the nonreducing-end of acarbose. The reducing-end of acarbose was modified by *Bacillus stearothermophilus* maltogenic amylase catalyzed transglycosylation reactions in which α-acarviosine-glucose from acarbose was transferred to a wide variety of acceptors. The nonreducing-end of acarbose was modified by transglycosylation reactions of *B. macerans* cyclomaltodextrin glucanyltransferase catalyzed reactions between cyclomaltohexaose and acarbose to give G6, G12, and G18 attached to the C-4-OH. These two enzymatic techniques might be used sequentially or possibly even simultaneously to produce acarbose-based analogues, modified at both ends.

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Chapter 14

Maltooligosaccharides from Corn

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The conversion of cornstarch to maltooligosaccharides is an area of significant commercial interest. The production of maltooligosaccharides on an industrial scale has been practiced for over 30 years. The products are used in a variety of applications, which include the food and pharmaceutical industries. This article will focus on the commercial production, characterization and applications of this product. In addition, recent progress in this area will be summarized.

Introduction

Maltooligosaccharides are oligosaccharides primarily composed of α-1,4 linked glucose units. These oligosaccharides are produced from the hydrolysis of starch. Maltodextrins are a subset of maltooligosaccharides that were defined by the FDA (*21 CFR 184.1444*) in 1983 as follows: "*Maltodextrin is a non-sweet nutritive saccharide polymer that consists of D-glucose units linked primarily by α-1,4 bonds and that has a dextrose equivalent of less than 20.*"

Therefore, the definition serves to carve out a specific molecular weight fraction of a starch hydrolysate as defined in the context of dextrose equivalence. Throughout this article, the term maltooligosaccharide will be used for general examples of starch hydrolysates that include maltodextrins, but will go beyond the dextrose equivalent barriers that define a maltodextrin.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

A generalized process flow chart for the production of maltodextrins is shown in Figure 1. The process involves either a one or two-step hydrolytic procedure in which starch is liquefied at high temperature via acid and/or enzymes to a desired final DE Further refining is necessary to remove insoluble solids, color and off-flavored material. The resulting syrup is then spray dried to a white powder.

The structure of a maltooligosaccharide unit is shown in Figure 2. When cornstarch is used, approximately 24% of the maltooligosaccharides will be derived from amylose and 76% from amylopectin. Amylopectin is a high molecular weight, branched polymer that imparts approximately 3% α-1,6 glucose linkages into the maltooligosaccharide product. This branching can have important functional implications because generally more branching leads to higher water solubility of the resulting product.

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Functions and Applications

Maltodextrins are items of significant commercial interest. The commercial market for these products is in the order of hundreds of millions of pounds per year. The properties associated with maltodextrins include:

1. High solubility.
2. High viscosity.
3. Bland taste – low sweetness.
4. Non-hygroscopic.
5. Free-flowing powder.
6. Non-hazing solutions.
7. Low osmolality.
8. Readily digestible.

These properties translate to a number of useful applications in the food, pharmaceutical and industrial fields, which include the use of maltodextrins as:

1. Carrying agents.
2. Bulking agents.
3. Texture providers.
4. Spray drying aids.
5. Fat replacement products.
6. Freezing point depression control agents.
7. Viscosifier/bodying agents.

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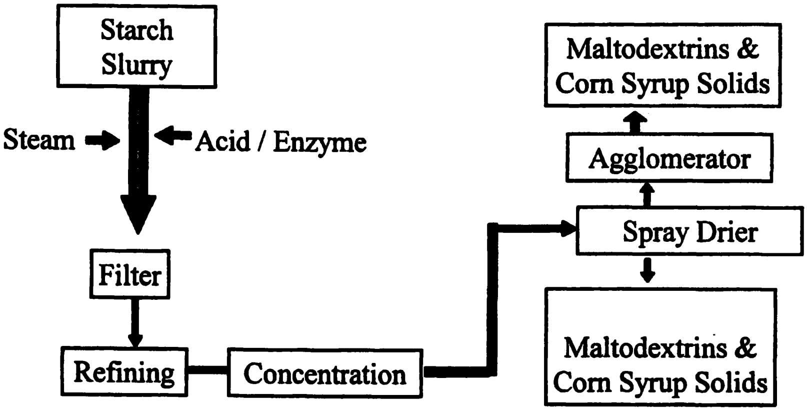
Starch Slurry
Steam
Acid / Enzyme
Filter
Refining
Concentration
Spray Drier
Agglomerator
Maltodextrins & Corn Syrup Solids
Maltodextrins & Corn Syrup Solids

Figure 1. Generalized maltodextrin process flow chart.

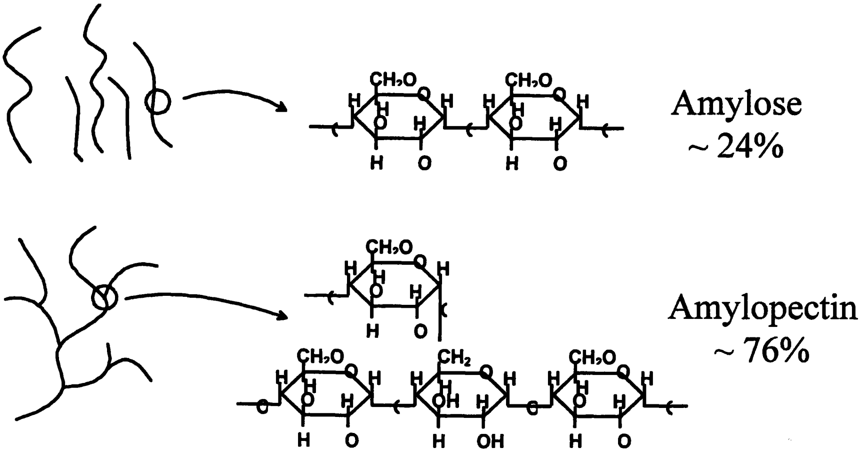
Amylose
~24%
Amylopectin
~76%

Figure 2. Amylose and amylopectin components of starch.

A qualitative measure of the ubiquitous nature of maltodextrins can be revealed by a trip to the local grocery store in which hundreds of products will list maltodextrin as an ingredient.

Characterization

Historically, dextrose equivalent (DE) has been used to describe maltooligosaccharides. DE is a quantitative measure of the degree of starch polymer hydrolysis. The higher the DE, the greater the extent of the hydrolysis. The older techniques used to measure DE are titrative methods based upon the reduction of cupric ion to copper (I) oxide by the free aldehyde group on the reducing sugars. Lane and Eynon (*1*), Schoorl (*2*) and Somogyi (*3*) are among the methods used to determine DE. For example, glucose would have a DE of 100, as every glucose unit is a reducing sugar capable of reducing Cu^{2+} to copper (I) oxide. Starch, with virtually no reducing sugars, has a DE of zero. Therefore a DE 10 material would have an approximate degree of polymerization (DP) of 10 units as only one tenth of the material can participate in the reducing reaction. Despite the heavy dependence on copper titrative methods to obtain DE, the methods overestimate the actual DE (*4-5*). Therefore newer methods, such as freezing point depression via osmolality measurements (*6-7*) have been developed that are more accurate and can obtain real time DE measurements.

Despite the widespread use of DE to obtain information about maltooligosaccharides, the method does not reveal any structural information that is necessary to study these molecules in more detail and develop new maltooligosaccharide based products. Therefore, more sophisticated techniques are routinely used to allow for examination of the chemical structure of maltooligosaccharides. Some of the techniques used include high performance liquid chromatography (HPLC), high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), capillary electrophoresis (CE), mass spectroscopy, and nuclear magnetic resonance spectroscopy (NMR).

HPLC

HPLC, and more specifically gel permeation chromatography (GPC), is one of the routine methods used to characterize maltooligosaccharides (*6*). This technique lends itself to the separation of the plurality of molecular species that comprise a particular maltooligosaccharide. One of the key measurements that GPC obtains is the degree of polymerization, or DP profile of a particular product. The DP profile can be used to obtain detailed information on how a

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variable change can affect the resulting maltooligosaccharide composition. However, GPC techniques that are practiced routinely in industrial laboratories tend to have good resolution up to a DP of about 10 units, which may not comprise the majority of the carbohydrate profile. A typical carbohydrate profile obtained via GPC is shown in Figure 3. Other GPC techniques such as HPLC-SEC TriSEC (*7*) can be used to obtain detailed information about the ultra high molecular weight component of a maltooligosaccharide mixture. Therefore, there remains a need to obtain better characterization of the maltooligosaccharide components between DP 10 and approximately DP 100.

HPAEC-PAD

High performance anion exchange chromatography with pulsed amperometric detection represents an improvement in the ability to resolve carbohydrates beyond a DP of about ten units. Typical chromatograms can sometimes obtain resolution beyond a DP of 30. One major drawback to this method is that it is difficult to obtain a quantitative measure of products from the PAD detector, as the appropriate standards that measure DP >10 are not readily available. An elegant technology has been developed (*8*) in which post column enzymatic hydrolysis can be used prior to PAD detection (HPAEC-ENZ-PAD) for accurate quantitation of maltooligosaccharides up to DP 77.

Capillary Electrophoresis

Capillary electrophoresis is another method that can be used to obtain good structural information about maltooligosaccharides. Routine resolution up to DP 100 can be obtained via this methodology. The technique suffers from the similar problems encountered with HPAEC-PAD in that quantitation of the higher DP units cannot be achieved via this technique. However, the resolution power is strong enough to obtain branching and isomerization information on molecular species of the same molecular weight.

There are a number of other methods that can be used to evaluate and characterize maltooligosaccharides. For example, mass spectral and nuclear magnetic resonance techniques are routinely used to obtain detailed structural information on maltooligosaccharides. However, the major intent of this article was to briefly touch upon some main characterization methods. This helps underscore the relatively high level of analytical sophistication required to not only characterize existing maltooligosaccharides, but to develop new maltooligosaccharide products.

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Recent Developments

Maltooligosaccharides are readily available, relatively inexpensive and are derived from a renewable resource such as corn. These three factors lead to a tremendous amount of interest in tailoring new maltooligosaccharide based products. The literature is rife with examples of modifications to maltooligosaccharides. Figure 4 represents a sampling of some of the modifications that have been used. This section has been written to give the reader an idea of some of the chemistries that can be applied in this area, and by no means is meant to be a comprehensive review of the maltooligosaccharide literature. The following reviews (*4-5,9-10*) help give the reader more information on the area of maltooligosaccharides and more specifically, maltodextrins.

The dominant philosophy behind the modifications of maltooligosaccharides is to impart more functionality into this class of molecule. This can be achieved primarily via three routes:

1. Chemical modification of existing maltooligosaccharides.
2. Modification of upstream processes to alter the carbohydrate profile to produce a novel maltooligosaccharide. This is most easily accomplished by the use of enzymes.
3. Modification of downstream processes to isolate and/or amplify a particular carbohydrate profile in a maltooligosaccharide mixture.

Chemical Modifications of Maltooligosaccharides

Chemical modifications of maltooligosaccharides is a field that is virtually unlimited in the number and types of modifications that can be applied to this class of molecule. The major constraint to the development of these products is in consideration of the economic issues, i.e. market applications, modification costs, regulatory constraints that will justify the production of a derivatized maltooligosaccharide. Typical chemical modifications of maltooligosaccharides (Figure 4) include polymerizations, reductions, etherifications oxidations, and esterifications.

Polymerization modifications to maltooligosaccharides include reactions in which the carbohydrate is either used as a scaffold or a pendant on a non-carbohydrate, larger molecular weight scaffold. For example, the grafting of a maltooligosaccharide onto an acrylate polymer is a good way of introducing properties such as charge or hydrophobicity to the carbohydrate moiety (*11-12*).

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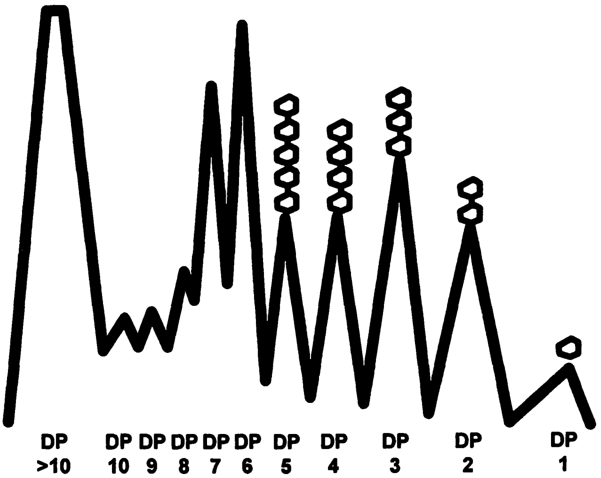

Figure 3. Typical maltodextrin carbohydrate profile. DP = Degree of polymerization

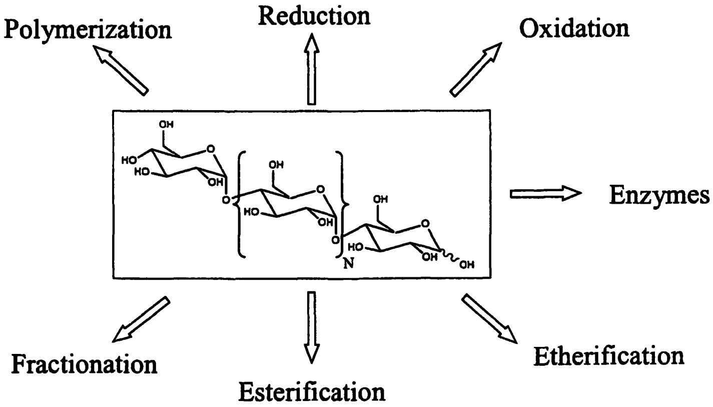
Polymerization
Reduction
Oxidation
Enzymes
Fractionation
Esterification
Etherification
OH
HO
HO
O
OH
O
OH
O
HO
OH
O
N
OH
O
HO
OH
OH

Figure 4. Methods to modify maltooligosaccharides

Alternatively, this grafting may also impart increased hydrophilicity and biodegradability into the non-carbohydrate portion of the polymer. Such modifications have importance in industrial applications such as paper production and detergent manufacturing.

Reductive chemistry has been used on maltooligosaccharides to produce new products that have enhanced biological, chemical and thermal stability. The most commonly used technique is catalytic hydrogenation with catalysts such as Raney® Nickel; however, the use of sodium borohydride has also been practiced (*13*). The resulting product is one in which the C-1 acetal functional group has been reduced to an alcohol group, in the same manner that glucose would be reduced to sorbitol. The use of reduced maltooligosaccharides as a starting material for further chemical modifications may be used due to their enhanced stability to alkaline conditions. Applications areas for reduced maltooligosaccharides, also referred to as hydrogenated starch hydrolysates (HSH) are very large and include food, pharmaceutical, and industrial areas.

Etherification chemistry has been used to modify maltooligosaccharides. Cationization, carboxymethylation, and hydroxyalkylation are all examples of modifications that have been done on these types of molecules (*14-15*). It must be noted that strongly alkaline conditions are necessary in order for these covalent modifications to occur and that these conditions usually lead to significant carbohydrate degradation.

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Oxidation of Maltooligosaccharides

Oxidation can be used to introduce carboxylate groups, and thus negative charge to maltooligosaccharides. Negatively charged polymers have numerous applications in the industrial, food and pharmaceutical arenas; thus an anionic product derived from a renewable resource would have significant commercial potential. The metal binding and water absorption properties that a negatively charged polymer would exhibit have applications such as for swelling agents, thickeners, binders, as well as for acidulants or complexing agents. The detergent industry would be an ideal market for an economical, environmentally friendly product with detergent building (i.e., calcium binding) properties that can replace existing polyphosphates.

A number of approaches have been applied to oxidize starch-based carbohydrates. The following reviews (*16-18*) can serve as a starting point for immersion into this subject. However, in general, many of the oxidation techniques that have been used, when applied to maltooligosaccharides, lead to depolymerization due to oxidation of the secondary hydroxyl groups. Therefore, technology has been developed to selectively oxidize only the primary position of maltooligosaccharides (*19*), and thus limit depolymerization. The use of TEMPO (2,2,6,6,-tetramethylpiperidine-1-oxyl) has emerged as one of the most efficient methods to selectively oxidize the primary position of a carbohydrate (*20*). De Nooy, Besemer and van Bekkum have pioneered the use of TEMPO for the oxidation of starch-based materials (*19,21-22*).

One of the most common TEMPO methodologies used to oxidize starch-based materials is shown in Figure 5. TEMPO, a nitroxyl radical, is used in catalytic quantities and is oxidized by a sodium hypohalite to the nitrosonium species. The nitrosonium ion, flanked by dimethyl groups, then is sterically limited to oxidizing only the primary positions on the glucose polymer, resulting in a glucuronate polymer. Conversion of the glucose polymer to over 95% glucuronate polymer is typical. The combination of sodium hypochlorite and catalytic sodium bromide has yielded the best results as hypochlorite oxidizes bromide ion to hypobromite, which then oxidizes TEMPO. One of the major drawbacks to this technique is that two equivalents of sodium hypochlorite are required for every primary hydroxyl equivalent. This leads to large quantities of sodium chloride that need to be removed from the final product.

The use of TEMPO-mediated oxidations as applied to maltooligosaccharides is described by Marsais *et al.* (*23*). The goal of this research was to develop a metal ion complexing agent that could be used as a detergent builder. It was found that when the TEMPO methodology was applied to lower molecular weight maltooligosaccharides, there was considerable depolymerization due to rapid oxidation of the hemi-acetal group. The resulting oxidized products also had sequestering properties that were significantly lower than commercial polyphosphate products.

Although TEMPO has emerged as an elegant oxidation strategy for starch-based materials, improvements to the existing methodology are necessary. Alternative co-oxidant / regenerant system using enzymes and / or TEMPO immobilization are examples of how this methodology will continue to improve with the goal of incorporating TEMPO in a commercial, large scale application.

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Esterfication of Maltooligosaccharides

Esterification chemistry is another commonly used modification that can be applied to maltooligosaccharides. The goal of this type of modification is consistent with trying to impart hydrophobicity and / or charge to the neutral starch hydrolysate. There are numerous types of chemistries that can be applied such as carbonate, phosphate, sulfate and boronate esterifications. For example, octenyl succinate (O.S.) can be used to derivatize a maltodextrin (Figure 6). The resulting O.S. maltodextrin has good emulsifying and encapsulating properties for food applications (*24*).

Another example of an esterfied maltooligosaccharide is a sulfation (*25*). This example was chosen as it represents how a modified maltooligosaccharide

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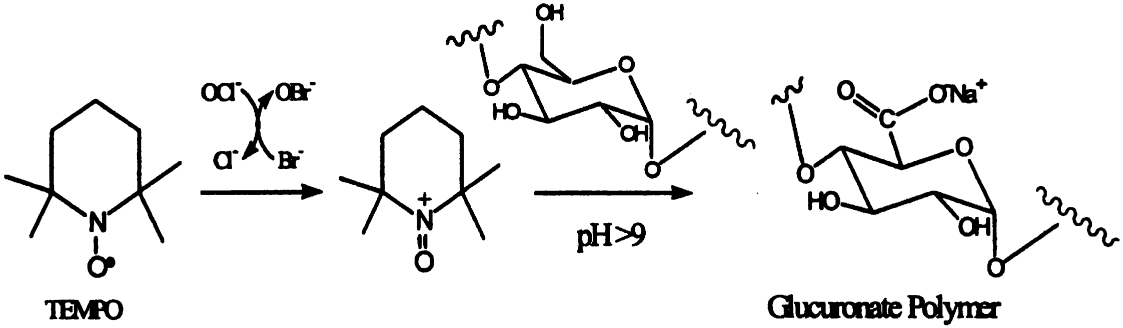
TEMPO
OCl⁻
OBr⁻
Cl⁻
Br⁻
pH>9
Glucuronate Polymer

Figure 5. TEMPO-catalyzed oxidation of D-glucans.

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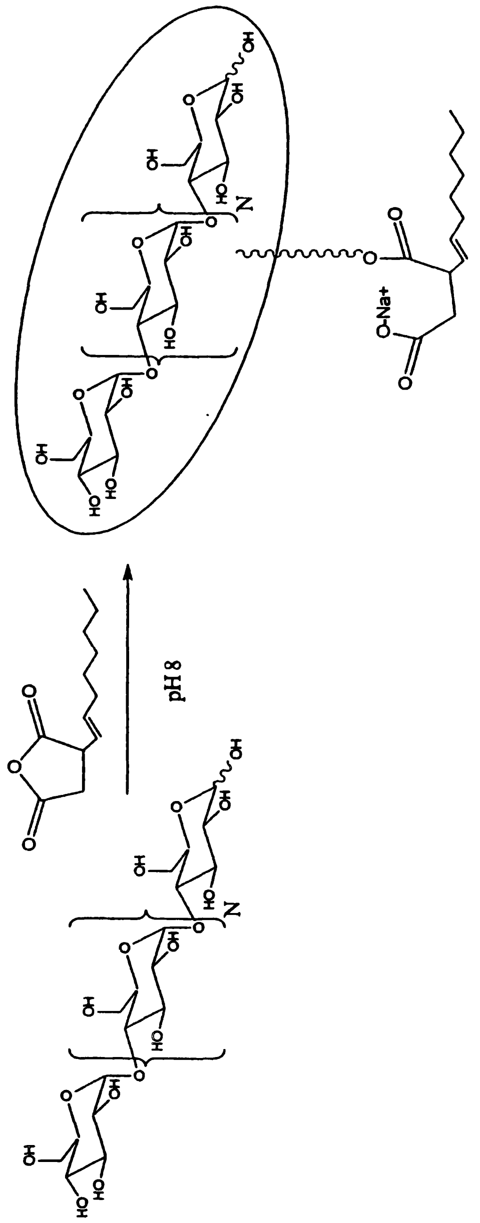

Figure 6. Octenyl succinate-modified maltooligosaccharide.

can be used in a biological application. It is known from the field of glycobiology that highly negatively charged carbohydrate biopolymers such as heparin, heparan sulfate and chondroitin sulfate are known to bind biologically active molecules such as selectins and basic fibroblast growth factors (bFGF). In this example, discrete maltooligosaccharides ranging from DP 1-7 were highly sulfated. The resulting anionic oligosaccharides were then shown to be able to block bFGF from binding heparan sulfate. The larger, more sulfated molecules showed the strongest effect. The implications of this research are that a modified maltooligosaccharide can be used as a relatively simple, pharmaceutical active.

Enzymatic Modifications of Maltooligosaccharides

One of the more active areas of starch chemistry is that of enzymatic modification of starches to yield new and unique maltooligosaccharides (*5*). The availability and discovery of enzymes that can either generate maltooligosaccharides of unique DP profile or operate under diverse hydrolytic conditions is driving this area. New discoveries in enzyme development have already been developed (*26*) and will continue to become sophisticated enough to produce maltooligosaccharides with highly defined DP profiles (i.e. maltotetraose or maltohexaose rich products). However, the need for more structure / function studies to better define commercially relevant applications is necessary to justify the research, development and commercialization of some of these new enzymes.

Maltooligosaccharides Generated via Downstream Processing

Downstream processing, such as membrane filtration, solvent precipitation and chromatographic separation can be used to generate maltooligosaccharides with unique carbohydrate profiles. For example, membrane filtration can be used to remove high molecular weight species from a maltooligosaccharide mixture. The resulting maltooligosaccharide product would have more haze stability and would be a good candidate for a liquid maltodextrin product (*27*). Filtration or yeast fermentation to remove the lower DP fraction of a maltooligosaccharide mixture is another example of downstream processing to create, in this case, a dextrose free product.

Conclusion

This manuscript serves as a brief introduction into the area of maltooligosaccharides. It is clear from the size and scope of the

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maltooligosaccharide market that this is a class of product that has had significant industrial importance for a number of years. Progress made in the characterization of maltooligosaccharides will continue to grow in sophistication as more information on the structure / function / application relationships of these molecules will drive the development of new products. The fact that maltooligosaccharides are readily available, relatively inexpensive and are derived from a renewable resource will ensure that maltooligosaccharides and new products derived from them will remain industrially significant in the future.

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Chapter 15

Some Effects of Oligosacchardies on Sucrose Crystallization

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The class of sugars known as oligosaccharides has been shown to be the principal cause of crystal elongation in the South African cane sugar industry. No single compound is totally responsible, but the kestoses (fructosyl-sucroses) and theanderose (glucosyl-sucrose) play a dominant role in this sucrose habit modification. The presence of these oligosaccharides leads to exhaustion difficulties when raw cane sugar is refined. The associated reduction in crystallization rates, leads to excessively long crystallization times, which has economic importance. Oligosaccharides occur naturally in sugar cane, but their accumulation between harvesting and processing is the major source of potentially troublesome oligosaccharides. A variety of chromatographic techniques has been used to isolate and identify some of the oligosaccharides, whilst HPAEC has been used to measure these sugars in a wide range of sugar products. Pilot plant studies have been used to indicate the extent to which oligosaccharides are transferred to the sucrose crystal. Some oligosaccharides are evident even in the refined sugar product.

In Oligosaccharides in Food and Agriculture; Eggleston, G., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

The Sugarcane Crop and Sugar Production

Sucrose is obtained commercially from two very different plant sources, sugar beet and sugarcane. In South Africa sucrose is produced exclusively from sugar cane. Sugarcane is a giant perennial grass of the genus *Saccharum officinarum*. Modern hybridization has led to improvements in sucrose yield as well as increased tolerance to some environmental stress factors such as drought or frost.

Typically the stalk consists of about 15% insoluble fibre and about 85% solution made up of water, sucrose and other dissolved substances in about 70:13:2 proportion. The minor components include other sugars (fructose, glucose, oligosaccharides and polysaccharides such as starch), salts (largely potassium), organic acids (predominantly aconitic acid) and small to trace amounts of color compounds (chlorophyll, phenolic acids), waxes and proteins. This composition can be affected if cut cane is stored before processing.

The South African cane industry is located mainly on the Natal coast within about 50 km from the Indian Ocean with the cane belt running in a SW direction from latitude 28°S to 31°S and includes the world's most southerly located sugar factory and cane growing area at Umzimkulu. The harvest season starts in April or May and continues until January or February. Three-quarters of the production in KwaZuluNatal is rain-fed, although some areas are reliant on irrigation. In South Africa most of the cane is cut by hand using long cane knives and is transported to the factory as whole stalk. Sometimes the green cane is cut, but cane is generally burned before harvest to avoid problems with snakes and rodents and to remove leaves and trash so that cutting is easier. There are, however, considerable environmental pressures to reduce this practice.

Cane processing includes

- harvesting the cane
- extraction of sugar in cane juice (by milling or diffusion, with the latter being the almost exclusive practice in South Africa)
- clarification of juice with lime and heat to remove solid impurities, to inactivate cane enzymes and to prevent sucrose inversion by raising the pH
- concentration of juice to syrup by evaporation under vacuum
- crystallization of sugar from syrup
- separation and drying of crystals
- further purification by refining.

Crystallization is the main purification stage and, for historic reasons, is generally a two step process. Cut cane is a labile commodity and raw sugar is produced close to cane growing areas. This sugar is then further purified in refineries - either attached to the raw sugar factory or located elsewhere. The introduction of membrane filtration will probably diminish this distinction between raw sugar processing and refining.

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Sucrose Crystal Habit

The sucrose crystal structure belongs to the space group $P2_1$ *i.e.* sucrose belongs to the hemimorphic class of the monoclinic system and has a two-fold axis of symmetry coinciding with the b-axis (conventionally this is represented by the horizontal axis in the sugar industry). The fructose component lies essentially in the ac plane, while the glucose half is approximately in the bc plane. The b-axis is asymmetrical in length so that sucrose crystals exhibit a left and a right pole. Such an effect is known as polarity. Sucrose usually shows higher growth rates at the right pole and greater face development on the left pole (Figure 1).

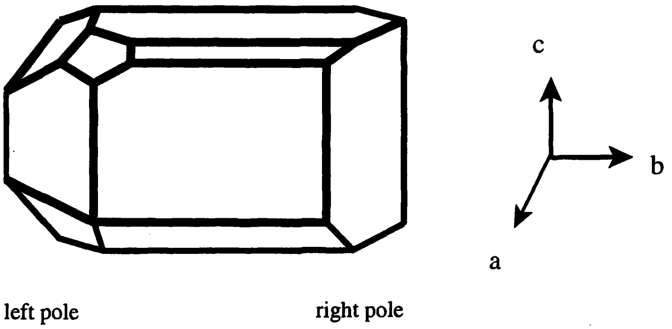

Figure 1. Schematic representation of sucrose crystal showing the most common faces (the angle ac is 103°).

The term "crystal elongation" is used to describe the shape of a crystal when the ratio of the crystal lengths in any two specified directions is different from that usually observed under similar crystallization conditions. In the cane sugar industry the c/b ratio is used as a measure of crystal elongation. Sucrose grown in pure aqueous solution is approximately twice as long in the b-axis direction as it is in that of the c-axis *i.e.* any sucrose crystal with a c/b ratio greater than 0.5 is elongated in the direction of the c-axis. The greater this ratio, the greater the extent of elongation. The general outward appearance of the crystal is governed by conditions during growth which can affect the growth rates of the different crystal faces relative to each other. Several factors can influence these growth rates - both physical and chemical *e.g.* temperature, viscosity and type and concentration of impurities. Under industrial conditions, cane sugar crystals frequently exhibit unusual habits such as fencepost, triangular or D-shapes. Needle-shaped crystals (showing b-axis elongation) are typical of low grade beet sugar crystals.

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Industrial Implications of Unusual Sucrose Habit

Crystallization is the main purification procedure used to recover sucrose from the sugarcane plant extract. Factors which affect the efficiency of this operation can have implications for crystal recovery (yield). Sucrose habit modification is linked to reduced crystal growth rates. This slows down industrial sugar production, effectively reducing factory capacity and leads to increased energy costs because of the longer processing times. In general, lower purity refinery products tend to give the most elongated crystals with elongation (and evaporative crystallisation times) increasing quite dramatically as the massecuite (*i.e.* the mixture of crystals and mother liquor) purity drops. These long thin crystals tend to be fragile and break easily. The smaller fragments can then pass through centrifugal screens - increasing losses to molasses and further decreasing recovery (yield). The loss of sucrose in final molasses (both physical and solubility effects) is the highest single loss in cane sugar mills (*1*).

Factors Contributing to Unusual Sucrose Habit and Reduced Growth Rates

An experimental program was introduced to pin-point the causes of the observed industrial crystal elongation.

Materials and Methods

Laboratory experiments were carried out at temperatures and saturations similar to those encountered in the factory. The sucrose/water (S/W) ratio was kept constant at 3.075 (supersaturation of 1.06 for pure sucrose), temperature at 60.5 °C. Rotation speed was constant and the non-sucrose/water (NS/W) ratio varied from 0.05 to 0.60. The solutions were doped with various selected impurities. Image analysis was used to measure the c/b ratio for the various crystal crops. The procedure was shown to be reproducible (2). Since physical conditions were constant and controlled it was concluded that any differences in crystal habit were caused by the concentration and quality of the impurities.

Isolation of Impurity Classes

Alcohol precipitation was used to obtain crude mixtures of high and low molecular weight components from industrial streams (e.g. refinery or factory molasses). Classical carbon-celite chromatography was used to separate the mono- and disaccharides from the higher oligosaccharides. The effect of these different groups of components on crystal habit and growth rate was evaluated. It was found that both the shape-modifying and rate-retarding components were present in the oligosaccharide fraction. Re-combination of the fractions showed that there had been no significant loss of any elongating properties (Table I).

Table I. Effect of Molasses Fractions on Crystal Shape and Growth Rate

Fraction	*Crystal shape (c/b)*	*Growth rate (*10^5) (kg.m^{-2}.s^{-1})*
Sucrose	0.54	5.7
Molasses (unfractionated)	1.33	3.1
Ethanol:		
HMWt fraction	0.51	6.5
LMWT fraction	1.25	4.2
Carbon:		
Mono- +disaccharides	0.59	5.1
Oligosaccharides	1.11	2.2
HMWt + LMWt (recombined)	1.33	

The oligosaccharide mixtures obtained from fractionation on carbon columns have been scanned using Fractogel TSK HW 40 (S). The HMWt fraction (which was found not to affect crystal habit) was not retained (*i.e.* no significant concentration of compounds of molecular weight less than 7 000 was found in this fraction). The fraction causing habit modification appeared to contain only compounds with elution volumes similar to those of tri- and tetrasaccharides.

These early trials implicated oligosaccharides in crystal elongation. Hence, further investigations focused on establishing the source of these oligosaccharides and their contribution to processing problems.

Oligosaccharides

Obvious possible components of the oligosaccharide fraction include the isomalto-oligosaccharides (as dextran anabolic or breakdown products) and maltose

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homologues (as amylose anabolic or breakdown products). Crude extracts of each of these classes were added to sucrose as though the molasses oligosaccharide fraction consisted entirely of each class. The shape of crystals grown in the presence of either malto- or isomalto-homologues was very little different from that of pure sucrose. Neither category of compounds affected the crystal shape. Only the molasses oligosaccharide fraction had any c-elongating effect. However, the habit modifying properties of this oligosaccharide fraction were completely removed after invertase hydrolysis. Normal shaped crystals were obtained when sucrose solutions were spiked with the hydrolysate. A similarly buffered control showed minimal improvement in elongation (Table II). This implies that the elongation promoting factors are fructose containing oligosaccharides, probably sucrose derivatives (*2*).

Table II. Effect of Oligosaccharide Classes on Crystal Shape

Preparation	*Crystal shape (c/b)*
Sucrose	0.54
isomalto oligosaccharides	0.57
maltooligosaccharides	0.56
molasses oligosaccharides	1.11
molasses oligosaccharides after invertase hydrolysis	0.51

Although well over 20 oligosaccharides have been detected in cane juice or molasses using thin layer chromatographic profiling, generally only about eight PAD responsive components are readily detected in raw sugar. The major oligosaccharide peak has been shown to be theanderose, which has been shown to contribute to c-axis elongation under refining conditions (*3*). However, the kestose isomers (1-, 6- and neo-kestose) can be present at relatively high concentrations. The other four components are relatively insignificant. Anion exchange chromatography using two different sodium hydroxide concentrations (60 mM and 100 mM) with sodium acetate indicated that two of these minor components are probably raffinose and erlose and that isomaltotriose, isomaltotetraose and maltose are absent (Table III). Higher homologues would elute late with the isocratic conditions used. Typically commercial cane raw sugar contains theanderose (180 - 350 mg/kg), neo-kestose (50 - 250 mg/kg), 1-kestose (50 - 175 mg/kg), 6-kestose (0 - 200 mg/kg), erlose (tentative) (50 - 100 mg/kg) and traces of raffinose. Papageorgiou and Doherty (*4*) using a 150 mM sodium hydroxide based solvent have also recently suggested that raffinose is present in the crystal.

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Table III. HPAEC Behavior of Minor Oligosaccharides in the Sucrose Crystal

Peak	R_{Raf}		*Comments*
	60 mM NaOH/ 10 mM NaOAc	*100 mM NaOH/ 15 mM NaOAc*	
1	1.000	1.000	raffinose, not isomaltotriose
2	1.620	1.392	not maltose
3	1.682	1.576	not maltose
4	1.900	1.805	probably erlose, not isomaltotetraose
Raffinose	1.000	1.000	
isomaltotriose	1.161	1.000	
maltose	1.774	1.432	
erlose	1.900	1.800	
isomaltotetraose	1.978	1.739	
maltotriose	6.035	4.278	

Crystal Transfer

Pilot plant crystallizations were used to show the selective incorporation of some impurities in the crystal (*5, 6*). Impurity transfer to the crystal will depend on many factors, but the controlled conditions maintained in the pilot plant allowed a comparison of the relative transfer of various impurities. As the concentration in the feed liquor increased, so did the level in the crystal. However, it can be seen that even when large amounts of monosaccharides are present in the syrup feed, their transfer to the crystal is clearly much less than that of the oligosaccharides (Figure 2). Moreover, as shown in Table IV, some oligosaccharides are more strongly adsorbed than others. A partition value defined as the ratio of the impurity concentration in the crystal to that in the feed liquor relative to refractometric dissolved solids (RDS) has been used to estimate the extent of transfer. The major oligosaccharides seen in the crystal are those most strongly transferred - theanderose, 6- and neo-kestose. The concentration of 1-kestose in the syrup is generally higher than that of the other oligosaccharides so that, despite its lower affinity for the crystal, it can be present at similar concentration in the crystal.

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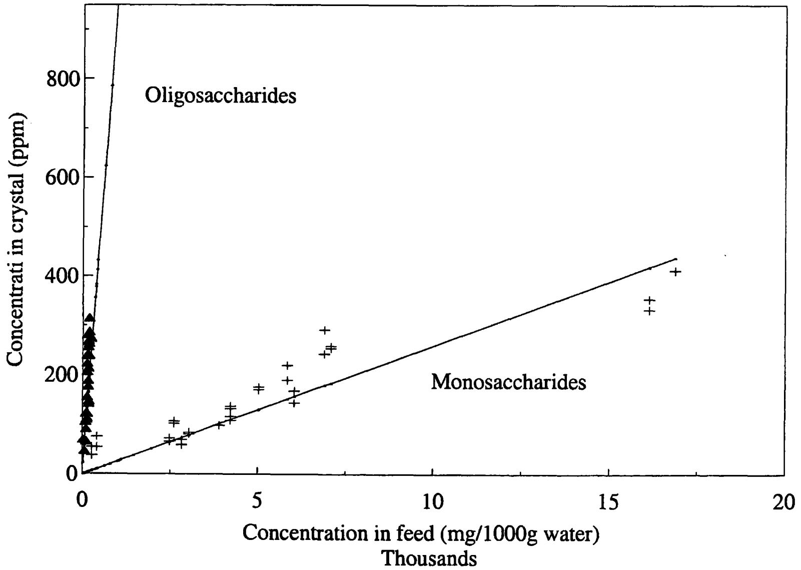

Figure 2. Transfer of monosaccharides (+) and oligosaccharides (▲) from mother liquor to the crystal during crystallization.

Table IV. Relative Transfer of Sugars to the Sucrose Crystal

Sugar	*Partition value*
Glucose	0.004
Fructose	0.005
1-kestose	0.049
6-kestose	0.143
neo-kestose	0.214
theanderose	0.252

Crystallization conditions will influence the magnitude of these partition values, but not the relative extents.

The oligosaccharides showing the greatest transfer are those having the greatest effect on habit modification and crystal growth rates. Smythe (*7*) found that particularly potent habit modifiers were those in which a hexose was substituted on carbon 6 of the glucose moeity of sucrose - raffinose, neo-kestose, theanderose, erlose for example. He postulated specific chemisorption effects on individual crystal faces being the reason for this habit modification.

Recently, Vaccari *et al.* (*8-10*) have demonstrated that crystals grown in the presence of high concentrations of a commercial fructo-oligosaccharide mixture (Actilight P^{TM}) assumed triangular to rhombic habits. They used planar chromatography to show that, despite being a minor impurity in the commercial mixture, neo-kestose was the dominant oligosaccharide in the crystal. X-ray diffractometry was used to confirm the preferential adsorption of neo-kestose on the right pole, resulting in the profound habit modifications observed.

Influence on Refining

The quality of refinery melt will depend on the quality of the raw input. Certain oligosaccharides tend to transfer preferentially to the crystal so that the sugar entering the refinery is enriched with compounds that will prevent efficient crystallization. This enrichment is depicted in Figure 3 where visual inspection shows that most of the oligosaccharides present in factory massecuite are excluded

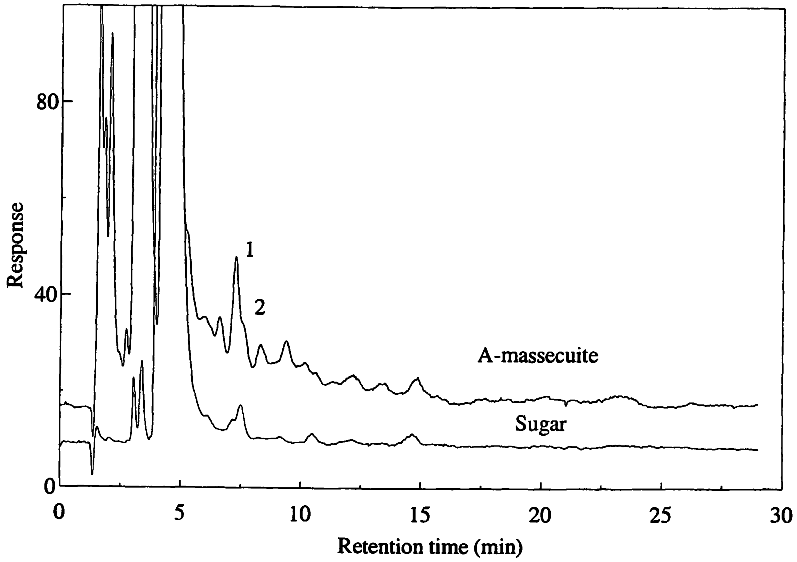

Figure 3. HPAEC profiles of oligosaccharides in A massecuite and A sugar (1 = 1-kestose, 2 = theanderose).

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from the crystal during crystallization as is to be expected, but relatively more theanderose than 1-kestose is transferred to the raw crystal. Theanderose has been found to transfer strongly to the cane sugar crystal and can be detected in the purest cane sugar (*11*). Beet and cane white sugar HPAEC profiles are shown in Figure 4. The raffinose peak is typical of beet sugars, whereas the theanderose peak has been found to be characteristic of cane sugars.

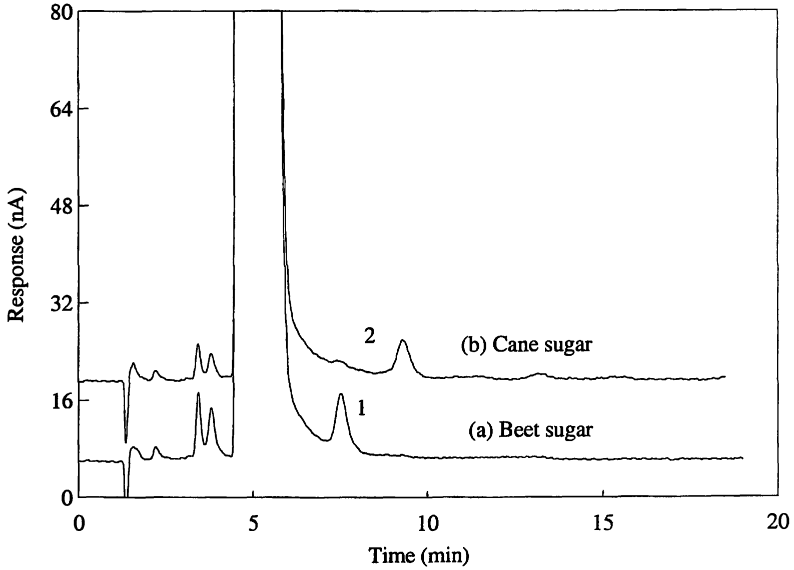

Figure 4. HPAEC profiles of cane and beet sugars (1 = raffinose (370 ppm); 2 = theanderose (345 ppm).

Source of Oligosaccharides

Oligosaccharides occur naturally in the cane plant and the levels vary with variety, age and maturity. In South Africa most cane is still harvested as whole stalk rather than billeted. Some of this cane is burnt in the field before cutting, whilst some cane is cut green and most of the leaves are removed. Although minimum harvest to crush delays are desirable this is not always possible. Cane delays are usually associated with processing problems including crystal elongation. The formation of those oligosaccharides detected in the crystal was monitored as a function of cane delivery delay. Trials were conducted over excessively extended periods in order to enhance the effects measured (*6*). There were no obvious differences in the types of oligosaccharide found in freshly harvested or stale cane

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or other factory products. However, with cane delay the formation of kestoses (fructosyl-sucroses) increased and these were the predominant oligosaccharides in cane juice.

The comparison of the behavior of burnt or unburnt cane to cane delay is summarized in Figure 5. The main oligosaccharide formed in both burnt and trashed cane was 1-kestose. (Trashed cane is cane with the leaves and tops removed after harvesting). All three kestoses formed more rapidly in deteriorating burnt cane than in green cane subjected to similar post-harvest conditions. Eggleston *et al.* (*12*) have recently shown that oligosaccharide formation is even more rapid in billeted cane (both green and burnt) than it is in whole stalk cane.

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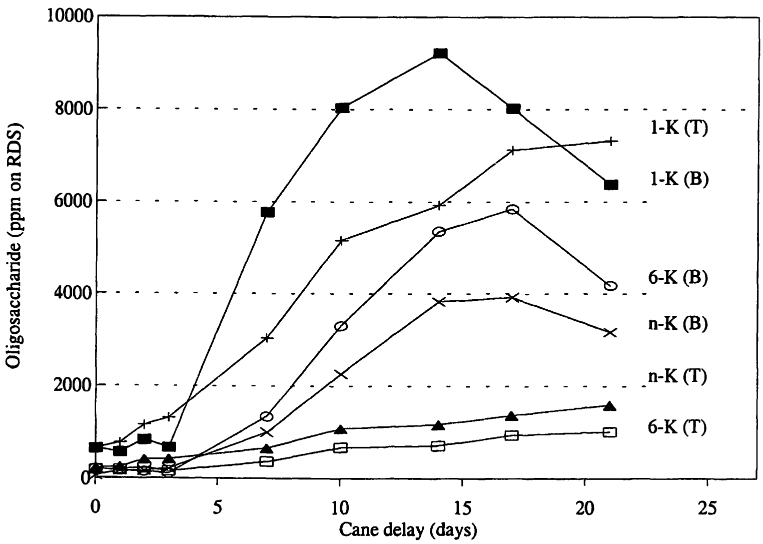

Figure 5. Concentration of kestoses in cane juice from deteriorating burnt or trashed windrowed cane. (1-K = 1-kestose; 6-K = 6-kestose; n-K = neo-kestose; B = burnt; T = trashed).

The main oligosaccharide detected in the crystal, theanderose (glucosyl-sucrose), appears to be a natural constituent of cane. There did not seem to be any appreciable change in its concentration during cane deterioration (a typical trial showing 240 mg/kg RDS (refractometric dry solids) on freshly cut cane with a maximum of 280 mg/kg RDS after a delay of 28 days) (*13*).

The greatest source of oligosaccharides in cane factories is as a result of post-harvest deterioration. This accumulation of oligosaccharides is only one manifestation of enzymic, bacterial or microbial activity. Cane deterioration will also be

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accompanied by the formation of other products such as acids and polysaccharides and environmental conditions (mechanical damage, cane maturity, temperature, humidity, rainfall *etc*) will dictate the major degradation pathways.

A further consequence of processing deteriorated cane is that additional kestose formation occurs during the extraction process. This can be avoided by the addition of a biocide (*e.g.* Busan 881). This is shown in Table V where the directly analyzed cane juice (a rapid homogenizer procedure) is compared with the composition of juice from a mill. This effect has been attributed to enzymic and microbial activity as a result of the ideal incubation conditions present during preparation and extraction of infected cane (*6*). In the absence of biocide the initial kestose concentration can more than double.

Table V. Comparison of 1-Kestose Concentrations for DAC Extracts and Mixed Juice from Deteriorated Cane With and Without Biocide.

DAC extract (mg/kg RDS)	*Mixed juice (mg/kg RDS)*		*Ratio MJ/DAC*	
	no biocide	*+ biocide*	*no biocide*	*+ biocide*
620	1340		2.16	
1280		1275		0.996
2080	7710		3.71	
2300	9005		3.92	
2400	9850		4.10	
2970		2375		0.800
3265	9075		2.78	
3415		2620		0.767
3420	5350		1.56	
3460		3190		0.922
5730		4890		0.853
6370		6500		1.020
7100		6465		0.911

DAC = direct analysis of cane
MJ = mixed juice
RDS = refractometer dry solids

Conclusions

Some of the oligosaccharides present in sugarcane are dominant sucrose crystal habit modifiers. This leads to changes in the sucrose crystal habit which results in reduced crystallization rates. Since the crystal elongating properties could be removed by invertase hydrolysis, the causative oligosaccharides are substituted sucrose molecules. Size exclusion chromatography showed that these oligosaccharides were tri-or tetrasaccharides.

The main source of these oligosaccharides is as a result of the formation of kestoses when cut cane deteriorates. Burnt whole stalk cane deteriorated more rapidly than green cane. The level in juice could be enhanced by the method of extraction. Theanderose, a characteristic oligosaccharide in cane sugar, and contributing to habit modification, did not appear to increase as cane deteriorated.

Oligosaccharides in the raw sugar are transported to the refinery where they are rapidly concentrated, leading to a reduction in the sucrose recoverable as crystal. Theanderose is so strongly adsorbed that it has been detected in all cane sugar samples examined. Its presence can be used to differentiate cane and beet crystals. Increased oligosaccharide concentrations in cane juice, which occur mainly as a result of cane delays, have an effect on crystallization rates - both in raw sugar processing and refined sugar production. Impurities associated with cane processing cannot be avoided, but the effects of oligosaccharides can be reduced by minimizing cane delays and maintaining good mill sanitation.

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Chapter 16

Oligocaccharidies in Cane and Their Formation on Cane Deterioration

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Cane deterioration in the field, factory storage pile, or during factory milling processes has become a major technical concern in recent years, especially in those areas of the world where mechanical harvesting of billeted sugar cane has increased dramatically. Not all deterioration products advocated as cane deterioration indicators impact future factory processing, but certain oligosaccharides directly impact processing by interfering with sucrose crystallization, deforming the crystal shape. Oligosaccharides are present in the cane before deterioration, with the tops (leaves and growing point regions) of the cane containing markedly more maltose, 1-, 6-, and neo-kestoses than the stalks. Chemical, enzymatic, and microbial reactions are all involved in cane deterioration and can cause the formation of oligosaccharides. The major oligosaccharides formed on cane deterioration are: 1-, 6-, and neo-kestoses (GF_2), nystose (GF_3), and kestopentaose (GF_4) isomers, as well as oligosaccharides formed as acceptor products from the action of dextransucrase in *Leuconostoc* bacterial strains, mainly isomaltotriose, isomaltotetraose, leucrose, and palatinose. Mannitol is also formed on cane deterioration by mannitol dehydrogenase also from *Leuconostoc* bacteria, and its rate of formation is higher than for oligosaccharides or ethanol and it is known to reduce sucrose recovery on processing. This paper reviews the effect of cane harvest method and storage time on oligosaccharide formation, as well as the use of HPAEC-PAD to simultaneously detect oligosaccharides and oligosaccharide isomers, mannitol, and ethanol - all cane deterioration products.

U.S. government work. Published 2003 American Chemical Society

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ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

Cane deterioration in the field, factory storage pile, or during factory milling processes, has become a topic of major concern in recent years, particularly in the U.S. where mechanical harvesting of billeted sugar cane has increased dramatically. Sucrose degradation reactions in cane deterioration include chemical (acid) and enzymic inversion reactions, and those from microbial activity, and can be influenced by cane health and environmental conditions.

A variety of cane deterioration products (*1-4*) have been used to confirm cane deterioration and delay (cut-to-crush time), and to predict and control processing problems at the factory. Such deterioration products have included high glucose and fructose concentrations, microbial (yeast, bacteria, and fungi) contamination (e.g., ethanol and lactic acid concentrations) and polysaccharides, but not all deterioration products affect future factory processes. Lionnet (*2*) stated that a cane deterioration product "will be useful only if it can be related to some aspect of the operations of the factory". Oligosaccharides are also products of cane deterioration (*1,4-5*) and certain oligosaccharides are responsible for crystal deformation problems (*1*). Ravelo *et al.* (*6*) reported that the formation of total oligosaccharides was greater than the formation of dextran and ethanol in cane subjected to delays and is, therefore, a more sensitive indicator of cane deterioration.

This chapter includes a study on the distribution of oligosaccharides in fresh cane tissue, and discusses the formation of oligosaccharides in cane deterioration under U.S. conditions, particularly Louisiana conditions. There is also a final section on oligosaccharides as sensitive indicators of cane deterioration that warrant further investigation for use at the factory.

Experimental

Chemicals and Reagents

HPLC grade sodium hydroxide and sodium acetate trihydrate were obtained from Fisher Scientific. Millipore™ water (18 M Ω) was used to prepare eluents and samples. Standard sugars, oligosaccharides, and sugar alcohols were analytical grade. Sodium azide was from Sigma. 1-Kestose, 1,1-nystose and 1,1,1-fructofuranosylnystose were from Dr. Takahisa Tokunage of Meiji Seika Kaisha, Ltd. Theanderose (6-O–α-D-glucosylsucrose) was from Wako chemicals and kindly purified by Dr. Greg Cote of USDA-ARS. Absolute ethanol was from Aaper.

Sampling - Oligosaccharides in Different Cane Tissues Study

Plants from two cane varieties, LCP 82-89 and LHo 83-153, were started in the greenhouse in the fall of 1997 and transplanted to the field on May 12, 1998.

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Variety plots were 3 rows (5.4m) wide and 5 m long, 36 plants per plot. The experimental design was a completely randomized block with six replications. Stalks were harvested from the first-ratoon and second-ratoon crops on 6 December 1999 and 11 November 2000, respectively. Twenty-one randomly selected cane plants were hand harvested from each plot. Senescent leaves were stripped from the stalks and discarded. Stalks harvested from the first-ratoon crop were separated into two sections, the mature stalk and the top containing the green leaves, the growing point region, and the immature internodes located just below the growing point. Juice was extracted from the stalks by passing the cane through a 3-roller laboratory sample mill, and from the tops with a Jeffco cutter grinder (Jeffress Engineering Pty Ltd, Australia) and a hydraulic press. Juice was subsequently analyzed for Brix (% dissolved solids) and oligosaccharides.

Stalks harvested from the second-crop were separated into four sections by dividing the top and stalk sections into two sections each. The upper section was divided into green leaves and the growing point region which included the upper immature internodes of the stalk. The mature stalk was divided into the basal six complete internodes and the remaining middle portion of the stalk. Juice was extracted from the two top sections with the cutter grinder and press, and the two lower sections with the roller mill. Juice from each of the four sections was analyzed for Brix and oligosaccharides.

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Sampling - Cane Deterioration Factory Study

This study was conducted between October 13-15, 1999, when weather and soil conditions were dry. Three Louisiana growers provided the harvested cane of variety LCP 85-384 to a Louisiana factory. On day 1, green billets were harvested by combine and delivered to the factory within 4h. Also on day 1, whole-stalk cane was hand-cut and cleaned (leafy trash material was removed) in the same cane supply and delivered to the factory. Later on this same day, standing cane was burnt along with cane laid into field heaps for later delivery on day 3, after 3 days of storage. On day 2, burnt billets were harvested by combine and delivered to the factory. On day 3, the 3-day-old-burnt whole-stalk treatment was delivered to the factory. On each day, except for the clean whole-stalk cane, seven to nine wagon loads of the appropriate cane supply were delivered from each grower to the factory. Core juice samples from each load were extracted in the factory core laboratory with a Cameco Core Sampler^{TM} using standard Louisiana factory procedures, then combined into three samples. Juice (200ml) had sodium azide (0.02%) added before being frozen in ice and transported quickly to the analytical laboratory. Samples were stored in a -40°C freezer until analyzed. The three juice samples from each grower were composited (or combined) before HPAEC-IPAD analyses.

High Performance Anion Exchange Chromatography with Integrated Pulsed Amperometry Detection for the Analysis of Carbohydrates and Sugar Alcohols

Carbohydrates (mainly oligosaccharides from 2 to 12 DP or degree of polymerization), sugar alcohols and ethanol were determined on duplicate juice samples. In order to compare chromatograms directly, all samples were Brix adjusted to the lowest sample Brix by adding water, before diluting then filtering through a 0.45 μm filter. For the cane tissue experiments the dilution was 1g/1ml, and for all the other deterioration studies the dilution was 1g/25ml. Carbohydrate HPAEC-IPAD chromatograms were obtained on a Dionex BioLC instrument. The carbohydrates and alcohols were separated on Dionex CarboPac PA-1 guard and analytical anion exchange columns (250 x 4 mm), at ambient temperature (~25 °C). Flow rate = 1.0 ml\min. Eluent conditions were: 100 mM NaOH isocratic (0.0-1.1 min; inject 1.0 min), a gradient of 0 to 300 mM NaOAc in 100 mM NaOH (1.1-40.0 min), and return to 100 mM NaOH (40.1-45.0 min) to re-equilibriate the column. Oligosaccharides and alcohols (from 100 μl injections) were detected with a PED-2 detector. The detector was equipped with Au working and Ag/AgCl reference electrodes operating with the following working electrode pulse potentials and durations: E_1=+0.05 V (t_0=0.00 s), E_2=0.05 V (t_1=0.42 s), E_3=+0.75 V (t_3=0.43 s), E_4=+0.75 V (t_4=0.60 s), E_5=-0.60 V (t_5=0.61 s), E_6=-0.60 V (t_6=0.96 s). Using a Spectra-Physics SP8880 autoinjector and Dionex Peaknet chromatography software, runs were accumulated of multiple samples and check standards. Oligosaccharides, mannitol, and ethanol were identified by comparing retention times with standards and by spiking with standards. Peak heights were measured to reduce the effect of interfering adjacent peaks. Mannitol, ethanol, palatinose, and leucrose were quantified in reference to mannitol, ethanol, glucose, and sucrose standards, respectively. Other oligosaccharides were quantitated in reference to raffinose.

Distribution of Oligosaccharides in Cane Tissue

The harvested sugarcane plant can be differentiated into distinct areas of tissue: the leaves, the growing point region, and the stalk (Figure 1). In order to elucidate the distribution of oligosaccharides in the harvested cane plant we used HPAEC-IPAD with a NaOH/NaOAc gradient method (see Experimental section) to separate and detect oligosaccharides and isomers in different cane tissue.

We first investigated the presence of oligosaccharides in stalks and tops (tops are defined as the leaves, plus the growing point region as well as the immature internode tissue just below it). The major oligosaccharides detected (Table I) were maltose (4-O-α-D-glucopyranosyl-D-glucose), 1-kestose (1^F-O–β-fructosylsucrose), 6-kestose (6^F-O-β-fructosylsucrose) and neo-kestose (6^G-O–β-fructosylsucrose). In plants, oligosaccharides can be classed as either primary or secondary oligosaccharides (*8*). Primary oligosaccharides are synthesized *in vivo* from a mono- or oligosaccharide and a glycosyl donor by the action of a glycosyltransferase.

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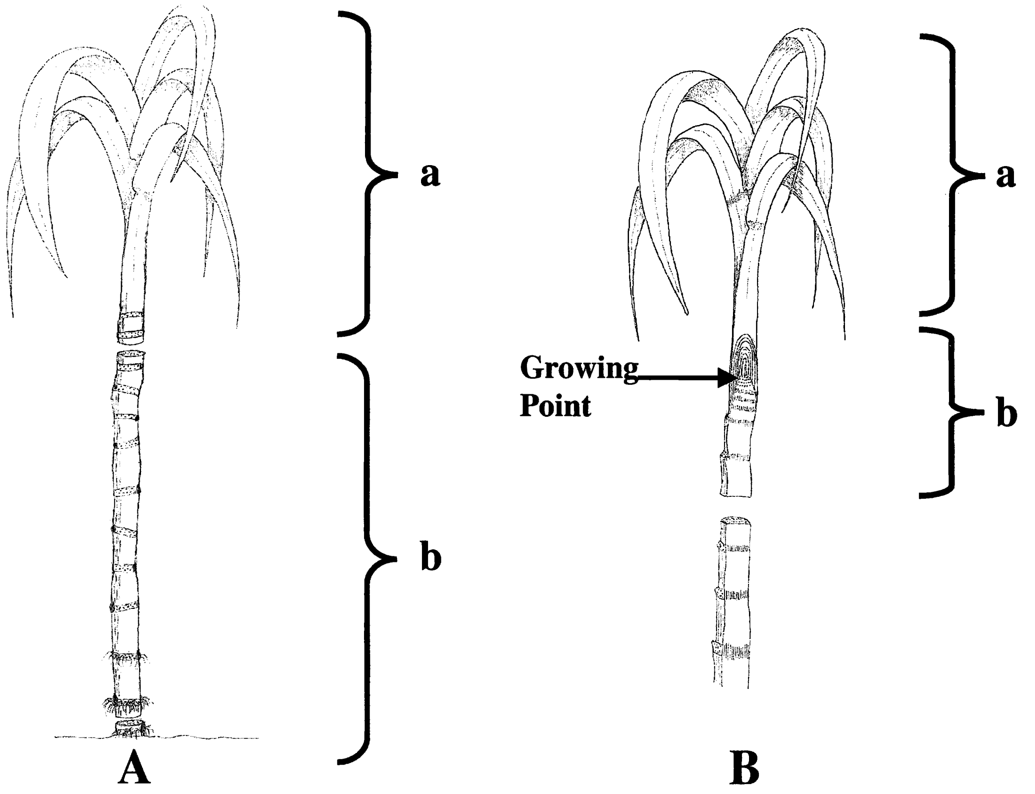

Figure 1. (A) Division of sugarcane plant into (a) the top with green leaves and (b) the mature stalk. (B) Top section of the plant divided into (a) green leaves and (b) the growing point region; the stalk was also divided into two other sections; the basal six internodes and the remaining middle stalk section (not shown). (Diagrams adapted from ref. 7.)

Secondary oligosaccharides arise from the hydrolysis of higher oligosaccharides, polysaccharides, glycoproteins and glycolipids *in vivo* or *in vitro*. Kestose GF_2 trisaccharides are known in C3 grasses and are usually secondary oligosaccharides formed from the breakdown of fructan polymers or higher oligosaccharide homologs, although in some plants they are not accompanied by higher homologs (8) and are, therefore, primary oligosaccharides. In sugar cane, a C4 plant, kestose GF_2 trisaccharides appear to be primary oligosaccharides as associated fructans derived from sucrose have not been reported, although a "fructan" that enzymatically breaks down into fructose and galactitol has been reported (*9*). The function of kestose trisaccharides in cane is still unknown; they may be storage carbohydrates or offer protection against freeze deterioration (*10*). 1-Kestose was the most abundant kestose trisaccharide isomer in both the stalk and tops, and there were markedly more kestose trisaccharides in the tops than stalks (Table I). This has important implications for factory processing as tops containing leaves are often delivered to the factory with associated green or unburnt cane stalks and are known as "trash", and kestose trisaccharides have been shown (*1*) to be responsible for deforming the crystal shape in the crystallization unit process. Trash contains other impurities such as high inorganic ash, color and polysaccharides which are also detrimental to processing.

Maltose is a secondary oligosaccharide in cane resulting from the breakdown of starch. Starch is a temporary storage polysaccharide in cane formed in the leaves via photosynthesis. It is usually broken down in the leaves during the night, and this explains the near eight-fold higher level of maltose in the tops than stalks (Table I).

Table I. Distribution of Primary and Secondary Oligosaccharides in Different Cane Tissue

	Oligosaccharide Concentrations ppm/Brix[a]			
Tissue	***Maltose***	***1-kestose***	***6-kestose***	***neo-kestose***
Stalk	103.5	227.5	62.2	49.0
Top	793.5	627.3	132.5	140.9

[a] Average of 3 replicates from two cane varieties, HoCP 85-153 and LCP 82-89

We further delineated the distribution of oligosaccharides in the cane plant by differentiating the cane tissue even further into leaves, growing point region (of the immature upper part of the stalk), middle stalk and bottom stalk (the basal six internodes of the stalk), which are illustrated in Figure 1. Example HPAEC-PAD chromatograms of the four different types of cane tissue are illustrated in Figure 2. In general, leaves and the growing point regions had markedly higher amounts of oligosaccharides (mainly 2 to 4 DP) than in either the middle or basal portions of the

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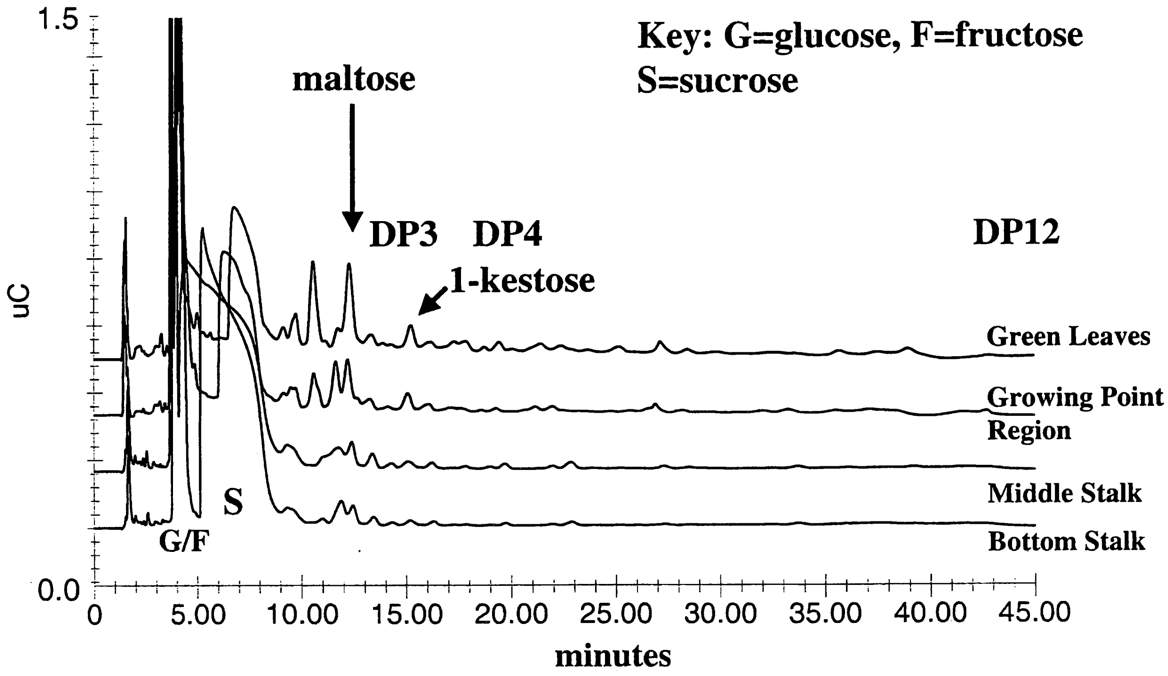

Figure 2. General distribution of oligosaccharides in the harvested cane plant (Brix standardized)

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stalks, and this was observed in two cane varieties: LCP 82-89 and LCP 83-153. Furthermore, more oligosaccharides of greater concentration and molecular weights were detected in the leaves than the growing point region. This confirms that the tops which include both leaves and growing point regions, contain most of the oligosaccharides associated with fresh cane. This is not totally surprising as the leaves and growing point regions are more active physiologically, and have much more enzyme activity associated with them, particularly invertases (*11*). Invertases (β-fructofuranosidases) can catalyze transfructosylating reactions with sucrose if the sucrose concentrations are high enough, to produce various kestose oligosaccharides of which 1-kestose is the morst prevalent (*12-13*). Results also suggest that other transfructosylating enzymes may be present in the leaves and growing point regions.

Although it was previously known that there were greater concentrations of polysaccharides in the leaves and tops of sugarcane than in the stalks (*14*), to the best knowledge of the authors, this is the first time that it has been reported that oligosaccharides are more abundant in the leaves and growing point region of the sugarcane plant than in the stalk.

Where Cane Deterioration Occurs

Figure 3 generally illustrates where most cane deterioration occurs, under U.S. conditions, in the field, and between the field and factory. In the field, cane can be susceptible to diseases, pests, and weeds. The burning of cane standing in the field or of whole-stalk cane laid into field heaps, occurs to remove tops and leaves, i.e., trash, and is still a common practice. However, burning can rupture cells which accelerates deterioration and the rate of deterioration largely depends on the degree of burning. Another cause of deterioration of cane in the field is the effect of freezes. In the United States, and also many other parts of the world, field cane is susceptible to freezes and associated freeze deterioration. In Louisiana, this problem is so severe that the frequent winter freezes force the industry to adapt to a short growing season (7 to 9 months) and a short milling season (approx. 3 months).

How the cane is harvested and then stored before delivery to the factory has a major impact on deterioration (*3-4*) and this will be discussed in the next section. Once the harvested cane is transported to the factory, it is usually placed on factory storage piles before processing; however, prolonged storage in such piles, especially in wet and warm weather, accelerates deterioration, especially microbial deterioration. Deterioration and loss of sucrose still occurs across factory processing, however, this is much less than that which occurs between the field and factory (*15*).

Effect of Cane Harvest Method and Storage Time on Cane Deterioration

Field Study

The cane harvest method, the field storage method for harvested cane, and length of storage time all have a major impact on cane deterioration (*3-4*). Many parts of the world harvest cane as burnt or green (unburnt cane which still contains trash) whole-stalks. Up until the early 1990s, cane in Louisiana was being harvested with

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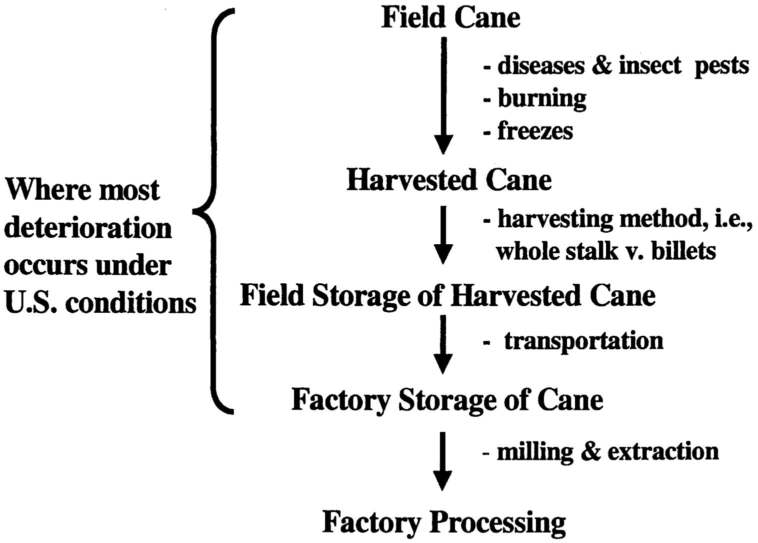
Where most deterioration occurs under U.S. conditions
Field Cane
- diseases & insect pests
- burning
- freezes
Harvested Cane
- harvesting method, i.e., whole stalk v. billets
Field Storage of Harvested Cane
- transportation
Factory Storage of Cane
- milling & extraction
Factory Processing

Figure 3. Where cane deterioration generally occurs under U.S. conditions.

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a mechanical soldier harvester to produce green or burnt whole-stalk cane. However, in 1993 a new, higher yielding cane variety LCP 85-384 was introduced to Louisiana but was found to lodge or fall easily and the soldier harvester is not able to harvest all the lodged cane, which minimizes yield return. In order to more efficiently harvest lodged cane, the combine harvester was introduced. Sugar yields per acre increased; however, the use of the combine harvester has also led to an increase in deteriorated cane being processed, because the multiple cut ends of the 15-21 cm billets provide greater opportunity for microbial invasion.

In a field study, Eggleston *et al.* (*3-4*) investigated the effects of eight different harvest methods and storage treatments on cane deterioration, with samples being taken on each day for four consecutive days (0 to 72 h) before laboratory juice extraction and analyses. Treatments included three that were handcut whole-stalk cane that was either hand stripped of leaves, was left unstripped (green), or was burnt. Five other treatments included three that were cut with a soldier harvester and two with a combine harvester. The soldier harvested whole-stalk cane was either burnt or green, or burnt and stored to simulate cane from a heap or transloader stack each day, with the latter treatment being the most prevalent whole-stalk harvest system in Louisiana. The burnt whole-stalk is harvested and laid into heaps on the ground and is often left for up to three days before transportation to the factory. Burnt and green billeted cane from the combine harvester were also taken, to simulate cane from a billet wagon each day. Billet wagons are often used in the field to store the billeted cane before transportation to the factory. It can be seen in Figure 4 that dextran formation, mainly by invading *Leuconostoc* lactic acid bacteria, was markedly greater and developed more rapidly in billeted cane than any of the whole-stalk cane treatments, with burnt billeted cane deterioration more rapid and extensive than in green billeted cane (*3*). Dextran is responsible for many of the numerous negative impacts that cane deterioration has on U.S. factory processing, mostly associated with the rise in viscosity from this polysaccharide.

The same HPAEC method (see Experimental section) used to differentiate oligosaccharides in the different cane tissue was used to determine the formation of oligosaccharides in the various cane harvest and storage treatments; example HPAEC chromatograms are illustrated in Figure 5. The changing oligosaccharide chromatogram profiles allowed for a much more sensitive elucidation of the different deterioration reactions contributing to overall cane deterioration. Using the HPAEC method it was possible to show that initially (0 h) there were no marked differences in oligosaccharide profiles for all the eight harvest treatments, which reflected the freshness of all the samples and indicated that, initially when the field cane is cut, freshness is more important than harvest method (*4*). This has considerable consequence for the factory in that as long as billeted cane is transported to the factory quickly on the day it is harvested, deterioration problems should be no worse than encountered in fresh-cut soldier harvested whole-stalk cane. There were, however, marked differences in the formation of oligosaccharides on storage/delay time. For the whole-stalk treatments shown in Figure 5, no significant increases in either kestose oligosaccharides or oligosaccharides formed by dextransucrase activity were observed on storage. In strong contrast, oligosaccharides formed rapidly (within 24h) on storage of both green and burnt billeted cane (Figure 5). Furthermore, greater deterioration occurred earlier and more rapidly in burnt than green billeted cane. The major deterioration oligosaccharides found in the billeted

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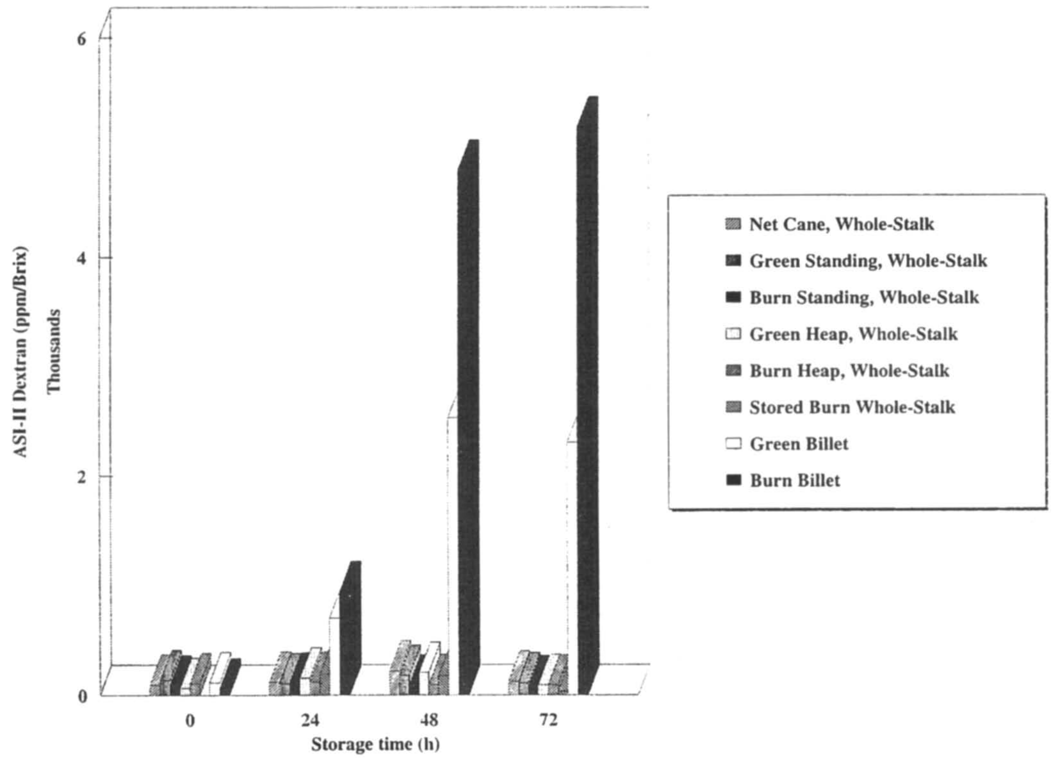

Figure 4. Effect of harvest method and cane delay on dextran formation (3,14)

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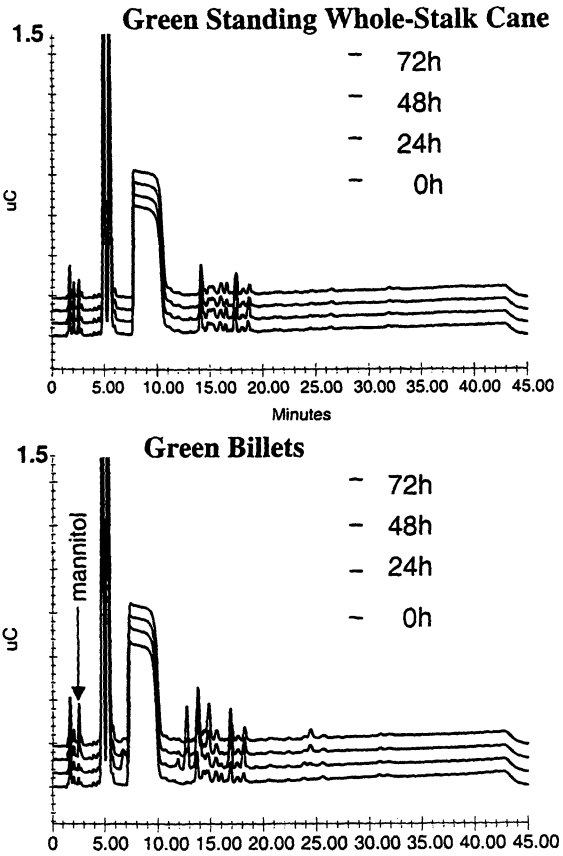

Figure 5. Example HPAEC chromatograms to illustrate the effect of harvest method and cane delays (Brix standardized)(4)

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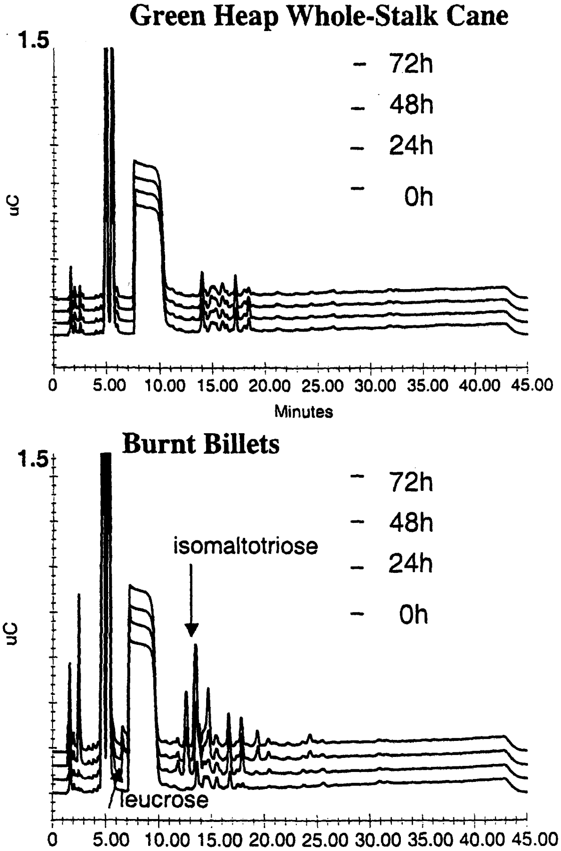
Green Heap Whole-Stalk Cane

Burnt Billets

Figure 5. *Continued*

cane included kestoses and oligosaccharide products of dextransucrase activity. The major oligosaccharides formed by dextransucrase in cane juice are palatinose (4-O-α-D-glucopyranosyl-D-fructofuranose), leucrose (5-O-α-D-glucopyranosyl-D-fructopyranose), isomaltotriose (α-O-D-glucosyl-[1→6]-α-D-glucosyl-[1→6]-D-glucose) and isomaltotetraose ((α-O-D-glucosyl-[1→6])$_3$-D-glucose) and these will be discussed in more detail in the final section of this chapter.

Overall, this study highlighted cane deterioration conditions in Louisiana which has a humid environment, and the real need to minimize harvest delays (cut-to-crush time) for mechanically harvested cane to reduce dextran and oligosaccharide loads at the factory, and prevent future processing problems. The latter is especially true for billeted cane, in particular burnt billeted cane, where there is a less than 24h time window from cutting-to-crushing. The four different cane growing areas in the U.S. (Louisiana, Florida, Texas, and Hawaii) are different environmentally. Consequently, although similar oligosaccharides most likely form during cane deterioration in these areas, the time-course of formation could be different in each environment. Furthermore, the distribution of microbes will also be different, which probably also affect how much of which oligosaccharides accumulate.

Factory Study

The HPAEC oligosaccharide profiles in the field study of cane harvest method and storage time stated in the last section (*3-4*), not only illustrated the marked differences in the rate of deterioration in whole-stalk versus billeted cane. They also demonstrated that if billeted cane could be delivered to, and processed at, the factory quickly (within 24h, but less than 14h is preferable [4]) no deterioration products should detrimentally affect processing. This important observation was reinforced by a further study at a Louisiana factory, where the delivery and factory processing of juice extracted from fresh green and burnt billeted cane was compared to 3-day-old-burnt whole-stalk cane and clean (stripped of leaves and green tops) whole-stalk cane. Typical oligosaccharide chromatograms for these juices are shown in Figure 6.

The major oligosaccharides observed in all four treatments were kestoses. There was no significant detection of oligosaccharides associated with dextransucrase activity, which indicates that very little microbial deterioration had occurred, and this was further confirmed by the similar size of the ethanol peak in each of the four treatments (Figure 6). Quantitation of the kestoses illustrated in Figure 7, further showed that the trisaccharide GF$_2$ kestoses, 1-, 6-, and neo-kestose, and to a lesser extent the GF$_3$ tetrasaccharide nystose (1,1-kestotetraose) were most abundant in the green billets which still contained leaves and tops. This confirms the previous results on the higher occurrence of these kestoses in the tops and leaves versus the stalks (see Table 1 and Figure 2). A DP5 oligosaccharide or pentaose was found to be more abundant in both the burnt billets and 3-day-old-burnt whole-stalk cane than the green billets and clean whole-stalks (Figure 6). By comparing the pentaose

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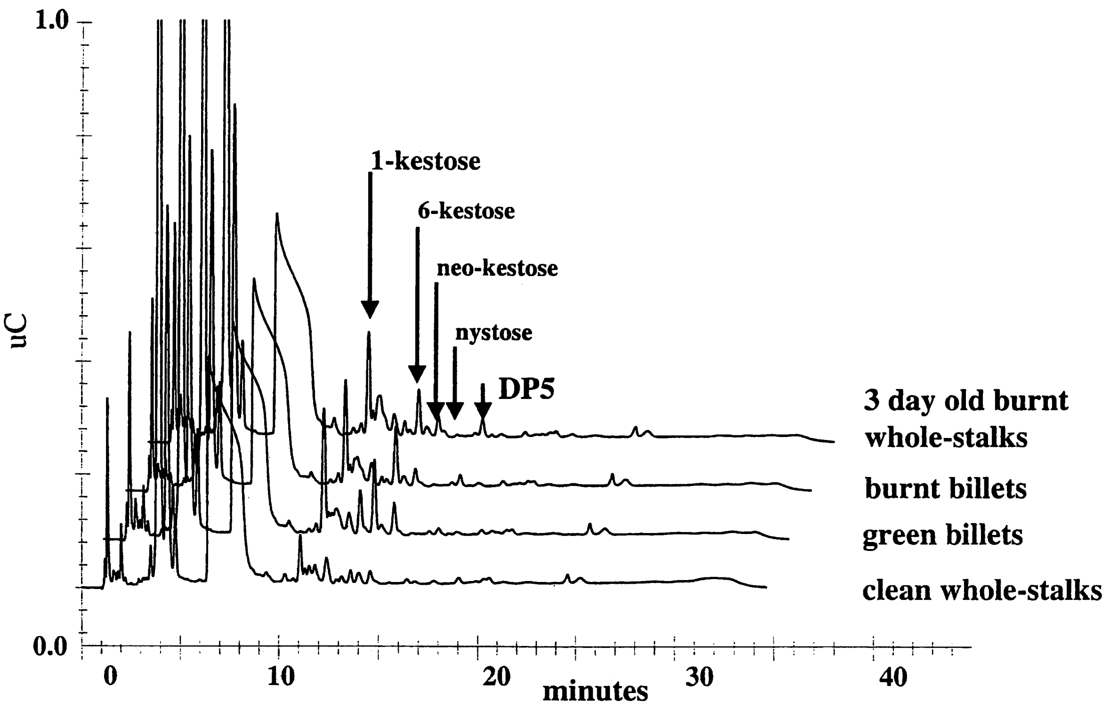

Figure 6. Effect of harvest method at the factory (Brix standardized)

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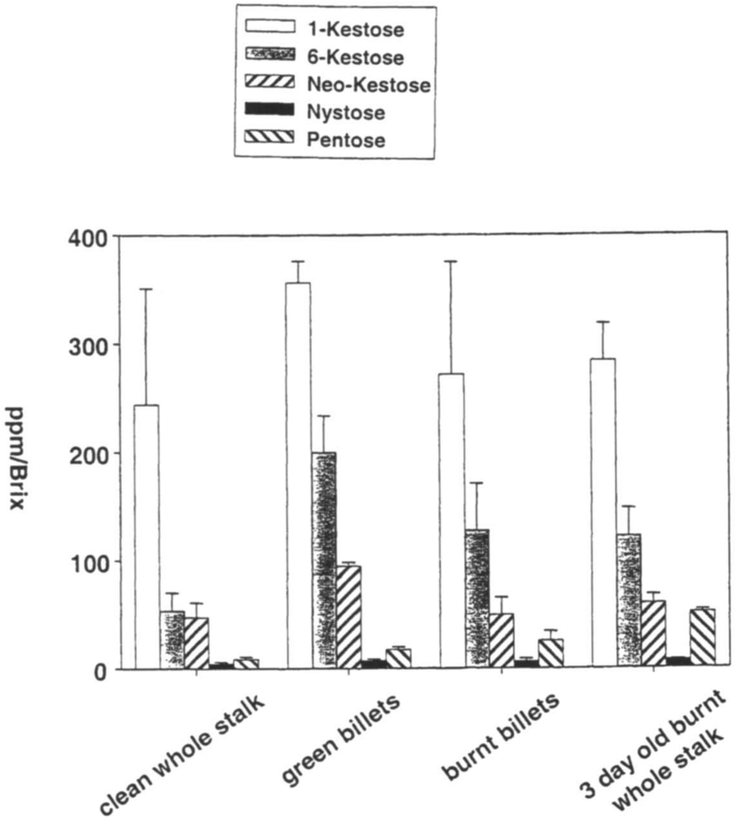

Figure 7. Effect of harvest method on oligosaccharide concentrations at the factory

retention time with a standard and by spiking with a standard, the pentaose has been tentatively identified as GF_4, 1,1,1-kestopentaose (1^F-fructofuranosylnystose). Furthermore, this pentaose consistently occurred as a major peak associated with some adjacent minor peaks (Figure 6), which are most likely the known isomers (*17*) of 1,1,1-kesto-pentaose. In a previous cane deterioration study Eggleston et al (*4*) tentatively identified kestopentaose isomer peaks that were also found to be greater in burnt billeted cane on storage than green billeted, although nystose was also found to be in greater amounts too.

Overall, this study confirmed that if either burnt or green billeted cane is delivered to the factory quickly after harvesting, dextran and oligosaccharide processing problems can be reduced or prevented. The study confirmed that green billets deliver more detrimental (*1*) kestose oligosaccharides to the factory than burnt billets or burnt whole-stalk cane.

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Sensitive Indicators of Cane Deterioration

Laboratory Study of Factory Cane Juice

Little work had been accomplished on accurately elucidating the relative contributions of microbial, enzymic, and chemical (acid degradation) reactions to sucrose lossses on cane deterioration at factory ambient temperatures. However, Eggleston (*18*) recently, by using biocide and heat treatments on a factory cane juice, observed that over the first 14h of deterioration, 93% of sucrose deterioration was microbial, 6% enzymic, and 1% chemical. This study (*18*) also identified deterioration products which can be used as sensitive indicators of deterioration of cane in the field and at the factory, and may be able to predict future processing problems. Eggleston (*18*) observed that the HPAEC method discussed in the previous sections of this chapter could be used to simultaneously detect oligosaccharides, mannitol (alditol), and ethanol cane deterioration products. The method, however, is relatively insensitive to ethanol, compared to mannitol, oligosaccharides, and other sugars. Hanko & Rohrer (*19*) used a HPAEC method to simultaneously analyze for sugars, alditols, and alcohols produced by growing yeast (*Saccharomyces cerevisae*) cultures.

Like dextran, mannitol is also formed by the lactic acid *Leuconostoc* bacteria (*20*), but unlike dextran which is formed by dextransucrase, mannitol is formed by mannitol dehydrogenase. Fructose, as an alternative electron acceptor, is reduced to mannitol by mannitol dehydrogenase (D-mannitol: NAD-2-oxidoreductase, EC 1.1.1.67). During this process, the reducing equivalents are generated by the coupled conversion of glucose into D(-)-lactic acid and acetic acid (*21*). Fructose can also be simply reduced to mannitol by mannitol dehydrogenase NADH linked activity in *Leuconostoc* bacteria (*22*). Mannitol was previously observed to be strongly correlated with the quality of frost-damaged sugarbeets (*23*) and, moreover, mannitol has been known to reduce sucrose recovery on processing (*24*).

Ethanol has also been advocated as a cane deterioration indicator (2), particularly in burnt whole-stalk cane (*25*). It is a metabolic by-product of many microbial reactions, and the amount formed depends on the type of microbe, as well as microbial growth parameters including temperature and humidity. Ethanol is a major by-product of yeast fermentation reactions, with yeast converting sucrose into ethanol and carbon dioxide, especially under dry and anaerobic conditions. It was stated by Mackrory *et al.* (*26*) that *Leuconostoc* bacteria, besides forming dextran, can also be heterofermentative and produce lactic acid, ethanol and carbon dioxide, although others (*27-28*) have reported that this is only the case if glucose, not sucrose, is the carbohydrate carbon source.

Figure 8 illustrates the changing HPAEC chromatograms on deterioration in an untreated cane juice. Even after the first 7 h, mannitol and isomaltotriose had increased, and leucrose was visible, confirming that mannitol dehydrogenase and dextransucrase activities were present (*18*). However, ethanol had also formed slightly and the °Brix had begun to decrease (Figure 8) which strongly suggests that *Leuconostoc* bacterial deterioration and other microbial (most likely yeast reactions are involved because of the reduction in °Brix) deterioration reactions were simultaneously occurring in the juice.

In another treatment, the juice was heated initially to dentature enzymes and destroy most microbes, and the effect of this pre-heat treatment is shown in Figure 9. In the pre-heated juice, none of the HPAEC deterioration products studied increased over the first 14 h (Figure 9), indicating that the initial heat treatment delayed deterioration (*18*). After 14 h, however, there was a marked increase in dextransucrase metabolites including isomaltotriose, isomaltotetraose, leucrose, and palatinose, which suggested that the sample had either been re-inoculated in the non-sterile incubator conditions or *Leuconostoc* bacteria growth recuperated (*18*). In addition to catalyzing the synthesis of dextran from sucrose, dextransucrase secreted mostly by *Leuconostoc* bacteria, also catalyzes the transfer of glucose from sucrose to other carbohydrates that are present in the cane juice. These carbohydrates are named *acceptors*, the reactions are *acceptor reactions* and the products formed are *acceptor products* (*29-30*). Isomaltotriose, isomaltotetraose, leucrose, and palatinose are the major acceptor products of cane deterioration. Isomaltotriose is an acceptor product of isomaltose (*29*), which in turn is formed from glucose as an acceptor. Dextransucrase catalyzes the transfer of glucose from the sucrose to the 6-hydroxyl group of the nonreducing-end glucose residue of the isomaltose and the first acceptor product formed in a series isomaltooligosaccharides (*30*) is isomaltotriose. The next product in the series is isomaltotetraose and so on until ~7DP. Therefore, as expected, isomaltotetraose was only detected after isomaltotriose (Figure 9). When fructose is the acceptor, leucrose and palatinose are formed depending on the ring form of the fructose (*30*). The major product leucrose is formed from D-fructopyranose, and the minor product palatinose is formed when D-fructofuranose is the acceptor. Consequently, as can be seen in Figure 9, leucrose formed earlier and more rapidly than palatinose.

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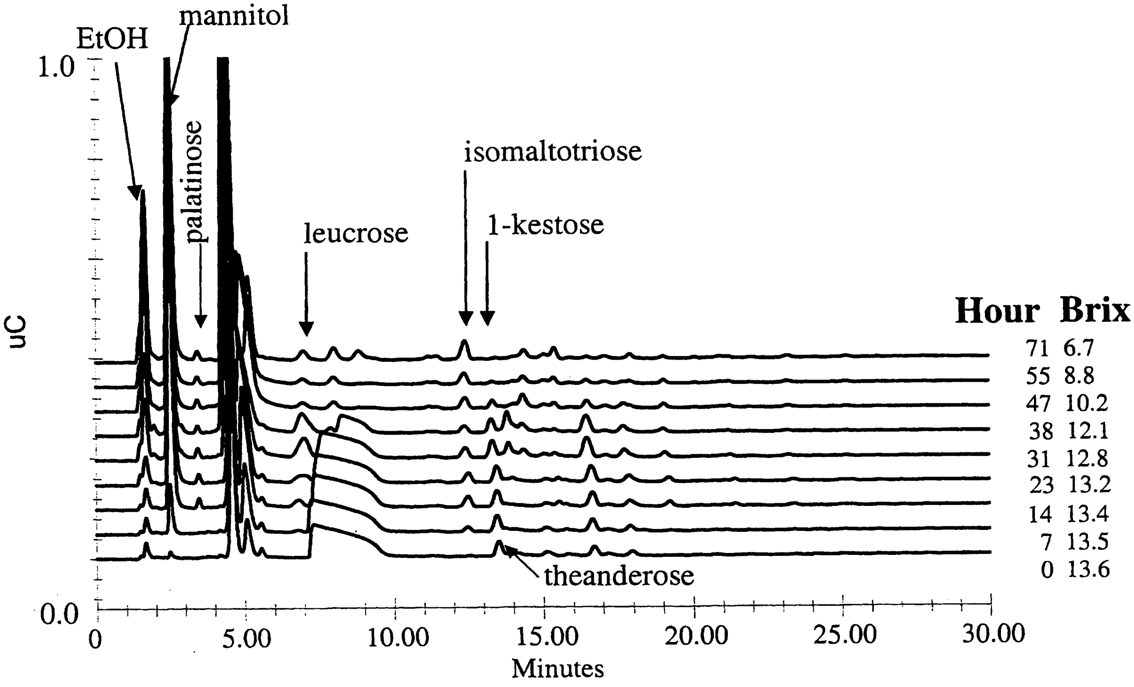

Figure 8. HPAEC chromatograms of untreated cane juice on deterioration (16)

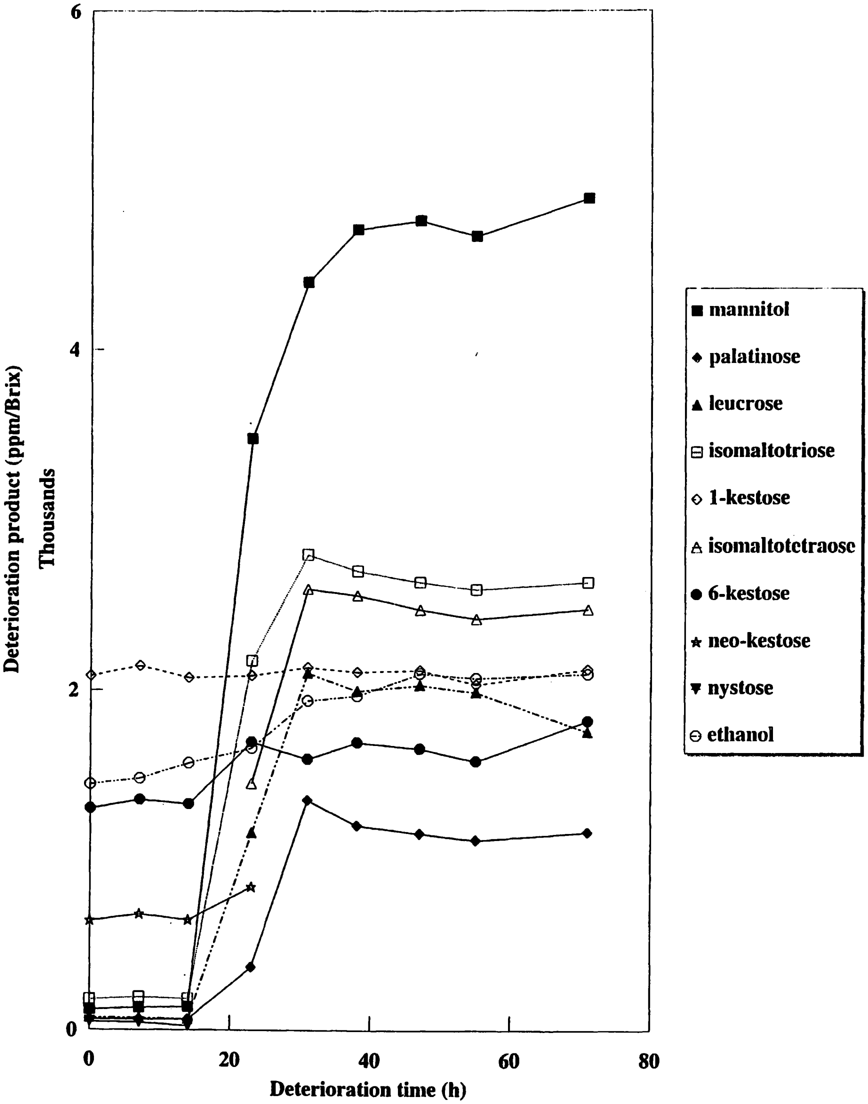

Figure 9. HPAEC deterioration products formed in initially heat-treated cane juice (16)

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In comparison to both leucrose and palatinose, the oligosaccharide iso maltotriose formed much more rapidly (Figure 9). This suggests that iso maltotriose is the most sensitive oligosaccharide indicator for dextran cane deterioration, which has also been recently further confirmed for cane freeze deterioration (*31*).

It can also be seen from Figure 9, that in strong contrast to the oligosaccharide acceptor products of dextransucrase, mannitol increased even more dramatically. As the rate of formation of mannitol was much higher than the other metabolites, including ethanol (Figure 9), it would be a very sensitive indicator for cane deterioration that could be used by factory staff to check if the load of cane delivered to the factory is going to cause dextran associated problems in subsequent processing. Moreover, there were excellent correlations between mannitol and dextran in the pre-heated (R^2=0.95, P<.005) and untreated (R^2=0.98, P<.015) juices (*18*), and further investigations on the use of mannitol as a cane deterioration indicator at the factory are being undertaken.

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Overall Summary

The U.S. sugarcane industry faces numerous challenges in the future. These range from changes in agronomic practices due to the introduction of new cane varieties and production management/harvest practices. Additionally, there are expected to be improvements in factory and refining processes. Oligosaccharides and other cane deterioration products detected by HPAEC-IPAD can provide us with an accurate and precise tool to monitor these changes and suggest methods for improvement.

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